Binary interactions between bacteria and *Candida albicans*

by

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Dissertation presented for the degree of
Doctor of Philosophy in the Faculty of Sciences at Stellenbosch University

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March 2017

Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes one original paper published in a peer-reviewed journal and two unpublished publications. The development and writing of the papers (published and unpublished) were my principal responsibility and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

March 2017 Eliska Benadé

Abstract

The primary habitat of the ascomycetous yeast *Candida albicans* is thought to be the mammalian gastrointestinal (GI) tract. This opportunistic pathogen however, was also found to be capable of sustainable growth in the anaerobic zones of fecal contaminated rivers. Although it is known that bacteria may impact the survival of *C. albicans* under aerobic conditions, the effect of an anaerobic environment on these bacteria/yeast interactions has never been explored. Therefore, using both aerobic and anaerobic liquid co-cultures, binary interactions between *C. albicans* and a number of environmental bacterial isolates were studied at 26ºC. The bacteria represented *Aeromonas hydrophila*, *Bacillus cereus*, *Bacillus subtilis*, *Clostridium, Enterobacter*, *Klebsiella pneumoniae, Kluyvera ascorbata* and *Serratia marcescens*. Using plate counts it was found that bacterial growth inhibits the yeast's growth under aerobic conditions; however, the inhibitory effect was ameliorated under anaerobic conditions. Bacterial enzymes capable of degrading yeast cell walls, including chitinases and mannanases, were produced by the majority of the bacteria. In contrast, except for *A. hydrophila,* these enzymes were not detected in anaerobic bacterial cultures, nor was the antimicrobial compound, prodigiosin found in anaerobic cultures of *S. marcescens.* Crude extracellular enzyme preparations from *K. pneumoniae* and *S. marcescens* had no effect on the yeast's growth, but were found to enhance the toxicity of prodigiosin towards the yeast, especially in combination with mannanases.

The above-mentioned *in vitro* methods were also used to study binary interactions at 37ºC between *C. albicans* and selected GI bacteria. The latter included *Bacteroides fragilis, Bacteroides vulgatus, Clostridium perfringens, Escherichia coli*, *K. pneumoniae, Lactobacillus acidophilus* and *Lactobacillus plantarum*. Using live/dead yeast viability staining in combination with epifluorescence microscopy it was demonstrated that the aerobic filamentous phase of *C. albicans*, which is usually induced at 37ºC, was either killed or inhibited by the bacteria; however, the bacteria had no effect on anaerobic yeast growth. Mannanase containing crude enzyme preparations from *K. pneumoniae* and *B. fragilis* also had no effect on anaerobically cultured yeast cells. Also, the enzyme preparations did not enhance the antagonistic effect of antimicrobials, such as Amphotericin B and prodigiosin, towards the yeast.

Generally, bacterial growth was unaffected by the presence of *C. albicans,* neither at 26 or 37ºC, nor under aerobic or anaerobic conditions. Under anaerobic conditions at 37ºC however, the presence of either *C. albicans* or the model ascomycete *Saccharomyces cerevisiae,* significantly increased the numbers of mannan utilizing *B. fragilis* and *B. vulgatus.* Conversely, this increase was not as pronounced in the presence of engineered *S. cerevisiae* strains with compromised cell wall mannan layers. Treating monocultures of the two *Bacteroides* species with intact dead yeast cells also resulted in significant increases in bacterial numbers, while removal of the cell wall mannan layers from these dead yeasts resulted in no increase in *Bacteroides* numbers. These findings indicated that mannan utilizing *Bacteroides* strains may form commensalistic interactions with *C. albicans*. Overall, our findings suggest that the symbioses between *C. albicans* and bacteria may differ depending on the presence or absence of oxygen.

Opsomming

Die soogdier se spysverteringskanaal word beskou as die primêre habitat van die askomisete gis, *Candida albicans*. Hierdie opportunistiese patogeen was egter ook al gevind om volhoubare groei in die anaërobiese sones van fekaal besmette riviere te handhaaf. Alhoewel dit bekend is dat bakterieë die oorlewing van *C. albicans* onder aerobiese toestande kan beïvloed, is die uitwerking van 'n anaërobiese omgewing op hierdie bakterieë/gis interaksies nog onbekend. Dus is beide aërobiese en anaërobiese vloeibare mede-kulture gebruik om binêre interaksies by 26ºC tussen *C. albicans* en 'n aantal omgewings-bakteriële isolate te bestudeer. Die bakterieë verteenwoordig *Aeromonas hydrophila*, *Bacillus cereus*, *Bacillus subtilis*, *Clostridium*, *Enterobacter*, *Klebsiella pneumonia*e, *Kluyvera ascorbata* en *Serratia marcescens*. Deur van plaattellings gebruik te maak is dit gevind dat bakteriële groei die gis groei onder aerobiese toestande inhibeer, maar nie onder anaerobiese toestande nie. Bakteriële ensieme wat instaat is om gis selwande aftebreek, insluitend chitienases en mannanases, was geproduseer deur die meerderheid van die bakterieë. In teenstelling, behalwe vir *A. hydrophila*, was hierdie ensieme nie gevind in anaërobiese bakteriële kulture nie, en die antimikrobiese verbinding, prodigiosin was ook nie gevind in anaërobiese kulture van *S. marcescens*. Ru ekstrasellulêre ensiem voorbereidings van *K. pneumoniae* en *S. marcescens* het geen effek op gis groei gehad nie, maar dit het wel die toksisiteit van prodigiosin teenoor die gis vermeerder, veral in kombinasie met mannanases.

Bogenoemde *in vitro* metodes is ook gebruik om binêre interaksies by 37ºC tussen *C. albicans* en geselekteerde spysverteringskanaal bakterieë te bestudeer. Laasgenoemde sluit in, *Bacteroides fragilis*, *Bacteroides vulgatus*, *Clostridium perfringens*, *Escherichia coli*, *K. pneumoniae*, *Lactobacillus acidophilus* en *Lactobacillus plantarum*. Die gebruik van lewendige/dooie gis-lewensvatbaarheidskleuring in kombinasie met epi-fluoressensie mikroskopie, het getoon dat die aerobiese filamentagtige fase van *C. albicans*, wat gewoonlik by 37ºC geïnduseer word, doodgemaak of geïnhibeer word deur die bakterieë. In teenstelling hiermee, het die bakterieë geen uitwerking op anaërobiese gis groei getoon nie. Ru-ensiem voorbereidings van *K. pneumoniae* en *B. fragilis* wat mannanase bevat, het ook geen uitwerking op anaërobies gekweekte gisselle gehad nie. Die ensiem voorbereidings het ook nie die antagonistiese effek van antimikrobiese middels, soos Amfoterisien B en prodigiosin, teenoor die gis verbeter nie.

Oor die algemeen was bakteriële groei nie geaffekteer deur die teenwoordigheid van *C. albicans* nie, nie by 26 of 37ºC of onder aerobiese of anaerobiese toestande nie. Alhoewel, onder anaerobiese toestande by 37ºC in die teenwoordigheid van beide *C. albicans* of die model askomisete gis, *Saccharomyces cerevisiae*, was die groei van beide *B. fragilis* en *B. vulgatus,* wat mannaan kan benut, aansienlik verhoog. Hierdie toename was egter nie so beslis in die teenwoordigheid van *S. cerevisiae* stamme met verswakte mannaan lae nie. Die behandeling van mono-kulture van die twee *Bacteroides* stamme met dooie gisselle het ook gelei tot aansienlike verhogings in bakteriële getalle, maar die verwydering van die mannaan lae van hierdie dooie giste het geen verhoging in *Bacteroides* getalle tot gevolg gehad nie. Hierdie bevindinge het aangedui dat *Bacteroides* stamme wat van mannaan gebruik kan maak, moontlik kommensalistiese interaksies met *C. albicans* kan vorm. In geheel dui ons bevindinge daarop dat die simbiose tussen *C. albicans* en bakterieë kan verskil afhangende van die teenwoordigheid of afwesigheid van suurstof.

Acknowledgments

My heart is overjoyed with thanks to everyone's love and support during this research over the years:

יהוה - For teaching me far more than the human mind can comprehend

''Set your mind on things above, not on things on the earth'' - Colossians 3:2

''He who dwells in the secret place of the Most High, shall abide under the shadow of the Almighty'' - Psalm 91:1

Pc Benadé – My husband for all his love and support, freshly roasted coffee and sarcastic puns always said at the right moment

My Family – Parents, sister and in-laws for all your support, love and encouragement

Prof A. Botha – A.K.A Doc, for his next level ideas during dream sessions, creativity, motivation, time, support, passion and many many laughs

Barbra Lerm – Future Dr. Toplis, my bestie from the lab. Thanks for all your love and endless laughter during the years

Dr. Marnel Mouton – My co-supervisor for all her input and advice

Lucky Mokwena – For putting in more effort than necessary in assisting with the GC-MS analyses

The National Research Foundation (NRF) and the Water Research Commission (WRC) for financial support that made this research possible.

“It's not that I'm so smart, it's just that
I stay with problems longer."

“Look deep into nature, and then you
will understand everything better.”

- Albert Einstein

Motivation and Project Aims

The ascomycetous yeast *Candida albicans* (Robin) Berkhout (1923) is a pleomorphic fungus and forms part of the normal microbiome of healthy individuals (Chester and Cooper 2011; Moran et al. 2012). It can live as a commensal in many anatomical sites within a mammalian host, such as the gastrointestinal (GI) and genitourinary tracts, oral cavities and skin of healthy individuals (Chester and Cooper 2011). When the immune system of the host becomes compromised however, the yeast is able to colonize and invade host tissue, resulting that this harmless commensal changes into an opportunistic pathogen (Wilson et al. 2012). *Candida albicans* is the most common human associated fungal pathogen (Beck-Sagué and Jarvis 1993; Chester and Cooper 2011) and is therefore the most studied of all *Candida* species.

Numerous studies implicated *C. albicans* as being responsible for the majority of candidiasis and candidemia cases around the globe (Guinea 2014; Pappas et al. 2003; Pfaller and Diekema 2007). A survey conducted in South Africa during 2014 and 2015 by the National Health Laboratory Service, Johannesburg, South Africa, revealed an overall incidence of 864 cases of candidemia in South Africa (GERMS-SA Annual Report 2015). *Candida albicans* was responsible for 42% of the cases followed by *Candida parapsilosis* with 26%. Of the 864 cases, a total of 24% were HIV-infected patients.

Even though mammalian hosts are generally believed to be the primary habitat of *C. albicans* (Moran et al. 2012), yeast strains are occasionally isolated from sewage sludge and waste waters contaminated with fecal material (Buck and Bubucis 1978; Cook and Schlitzer 1981; Cooke et al. 1960). In South Africa, populations with a high rate of HIV/AIDS infections often utilize polluted rivers for domestic purposes, such as ablution and drinking water (Paulse et al. 2009). Alarmingly, a study done by Stone et al. (2012) demonstrated that *C. albicans* is capable of growth along the banks of such a polluted river. Using quantitative real-time PCR they found indications that *C. albicans* is capable of sustainable growth in reducing, oxygen limited zones, while no evidence of such growth for this yeast was obtained in the more aerobic zones of the river. The reason for this phenomenon was still unclear.

Candida albicans is able to survive and proliferate in diverse environments within its host by overcoming challenges unique to certain sites (Kumamoto 2008). For example, this pathogen is able to withstand significant changes in oxygen and carbon dioxide levels, pH, osmolarity,

temperature and availability of nutrients. It is tempting to speculate that these characteristics would enable this yeast to thrive in external environments away from its human host. Hube (2004) stated that it is surprising that *C. albicans* is exclusively associated with animals or humans and hardly found in environmental niches such as soil, since it has no specific nutrient requirements that would prevent it from surviving in the outside environment. A well-known protective structure of yeast cells, which may contribute to this yeast surviving in external environments, is their robust cell wall (Bowman and Free 2006). Yeast cell walls play an essential role in protecting them from their surrounding environment, while the mannans in the outer layer of the cell wall show low permeability and porosity, thereby protecting the cell against antimicrobial agents (Gow and Hube 2012).

In the GI tract of humans, *C. albicans* population size is thought to be regulated by commensal bacteria (Kennedy and Volz 1985). In an environment such as a polluted river, many bacterial species may be present which can potentially have a negative effect on *C. albicans* growth (Britz et al. 2013). In addition, a wide variety of bacteria are known to be mycolytic and produce hydrolytic enzymes capable of degrading the different components of fungal cell walls (Azam and Malfatti 2007; De Boer et al. 2005). Hence, bacteria may play an important role in controlling *C. albicans* numbers within a polluted river environment.

Keeping the above in mind, the overall aim of this project was to study the interactions between bacteria and *C. albicans* under both aerobic and anaerobic conditions, and to investigate how the candidial mannan layer contributes to these interactions.

The first objective (Chapter 2) was to study binary interactions between strains of *C. albicans* and selected bacteria originating from the same sewage polluted river environment, under both aerobic and anaerobic conditions. Also, we aimed to screen the bacteria for enzyme production and test whether, in combination with an antimicrobial agent, mannan degrading enzymes exert greater synergistic antifungal activity against unicellular growth of *C. albicans* than other cell wall degrading enzymes such as chitinases.

The second objective (Chapter 3) was to study binary interactions between strains of *C. albicans* and bacteria associated with the mammalian host, under both aerobic and anaerobic conditions; and to determine whether selected antifungal drugs in combination with mannan degrading enzymes exert synergistic antifungal activity against unicellular growth of *C. albicans* than the drugs alone.

The third objective (Chapter 4) was to use *C. albicans* and the model ascomycetous yeast *S. cerevisiae,* to study binary interactions between these yeasts and *Bacteroides* species under anaerobic conditions. Also, we aimed to evaluate the role of the yeast cell wall mannans in these interactions.

This dissertation is presented as a number of chapters covering a review of the scientific literature relevant to the study topic (Chapter 1) and the research performed to meet the aim of the study (Chapters 2 - 4). General conclusions and future research are covered in Chapter 5.

* Please note, chapter 2 has been written in a style suitable for a scientific journal. As a result, repetition of some information could not be avoided and style may differ from other chapters.

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Chapter 1

Literature Review

1.1 *Candida albicans*

The ascomycetous yeast *Candida albicans* (Robin) Berkhout (1923) is a pleomorphic fungus (Chester and Cooper 2011). It is the most common human associated fungal pathogen (Beck-Sagué and Jarvis 1993; Chester and Cooper 2011) and is therefore the most studied of all *Candida* species. It was assigned to the genus *Candida* which was established to accommodate ascomycetous yeasts without a known sexual stage or other distinctive phenotypic characteristics (Tsui et al. 2008). Globally, this yeast is responsible for the majority cases of both candidiasis and candidemia (Pappas et al. 2003; Pfaller and Diekema 2007), followed by, *Candida glabrata* (Li et al. 2007). *Candida albicans* occurs as part of the normal flora in the human gastrointestinal (GI) and genitourinary tracts; however, when the immune system is compromised the yeast is able to colonize and invade host tissue, rendering it both a commensal and opportunistic pathogen (Chester and Cooper 2011). This yeast can grow under both aerobic and anaerobic conditions (Chester and Cooper 2011; Dumitru et al. 2004), thereby optimizing the number of niches it can colonize within its human host. The oxygen relations of this yeast however, is not the only trait that benefits its adaptability in the environment. A number of morphological features is known to equip this versatile yeast to adapt to changes in its physicochemical environment.

1.2 Morphology

1.2.1 Polymorphism

Candida albicans is often referred to as a dimorphic yeast, although in reality it is polymorphic, with several distinct morphological forms (Fig. 1.1). It occurs in at least three different forms: unicellular budding yeasts, pseudohyphae and true hyphae (reviewed by Whiteway and Bachewich, 2007). Yeast cells are spheroidal to ovoid in shape and reproduce via budding during vegetative growth. Pseudohyphal growth is believed to play a role in scavenging for nutrients away from the parental cell. Pseudohyphae comprise of elongated, ellipsoid cells which remain attached to one another at a constricted septation site, while typically proliferating in a branching pattern. True hyphal cells are long with parallel-sided walls and no apparent constrictions between cells. Occasionally, the term filamentous is used when it is not clear whether cells are hyphal or pseudohyphal.

In addition, to the above mentioned morphological feature, *C. albicans* is able to form chlamydospores (Chester and Cooper 2011). These spores are spheroidal thick-walled cells, three to four times the size of a regular vegetative yeast cells (Jansons and Nickerson 1970) and are characterized by a high lipid (Miller et al. 1974) and carbohydrate content (Fabry et al. 2003). Chlamydospores can be induced on specific nutrient-poor media, such as corn meal agar at lower temperature, while being incubated at lower than optimum growth temperatures, under oxygen limitation and low light conditions (Whiteway and Bachewich 2007). The presence of chlamydospores can be used to differentiate *C. albicans* from its close relative, *C. dubliniensis*. The latter is known to produce chlamydospores on Staib agar, while *C. albicans* is unable to do so (Staib and Morschhäuser 2007). A number of molecular studies were conducted to investigate gene regulation during chlamydospore formation (Alonso-monge et al. 2003; Nobile et al. 2003; Sonneborn et al. 1999). It was found that the transcriptional regulators Efg1p, the MAP kinase Hog1p as well as six genes are required for efficient chlamydospore formation. It is thought that chlamydospore formation occurs to ensure survival when exposed to harsh environments outside the human host, especially since these thick-walled cells have rarely been observed *in vivo* (Chabasse et al. 1988; Cole et al. 1991) and is known to be formed at lower temperatures. A well-known protective structure of the yeast cell however, playing a pivotal role in protecting the yeast from its surrounding environment, is its cell wall (Bowman and Free 2006).

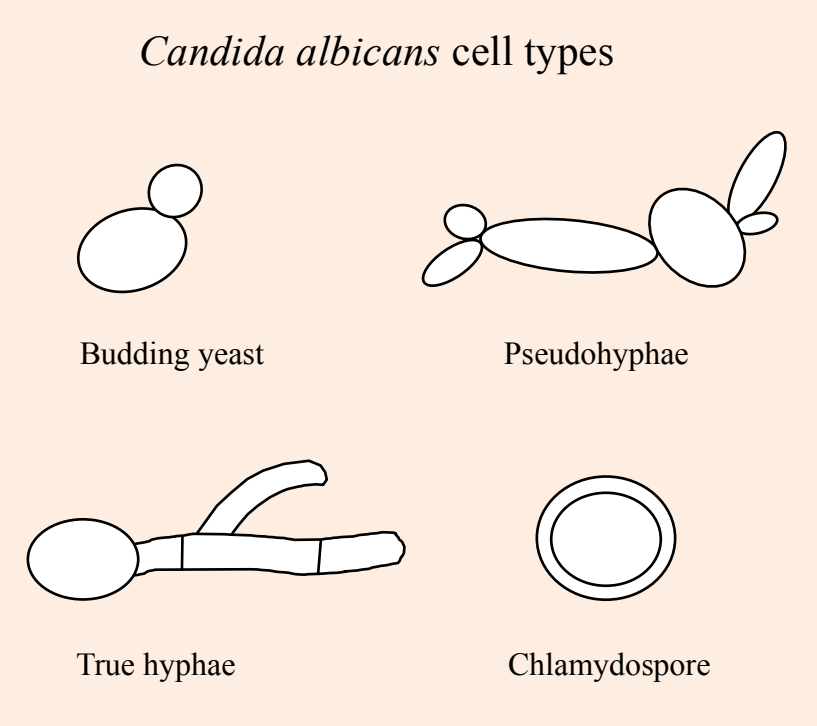


Fig. 1.1 The morphologically different cell types of *Candida albicans*.

1.2.2 Cell wall

The fungal cell wall functions as an exoskeleton and is essential for establishing and maintaining cell shape (Klis et al. 2002). These cell walls however, are also highly elastic and protect the cell against osmotic pressure and other environmental changes. Another function of this vital structure is to allow cells to interact and adhere to one another and external surfaces. In medically relevant fungi, cell walls mediate primary interaction between the cell and host tissue, permitting fungal cells to bind to, and in some cases invade host tissue (Zordan and Cormack 2012). Interestingly, fungal cells are known to respond to a variety of external signals which influence cell wall structure and organization (Munro and Richard 2012). In the experimental model yeast *Saccharomyces cerevisiae*, an estimated 1200 genes directly or indirectly affect cell-wall structure and organization (de Groot et al. 2001). One can speculate that this number may be even higher for *C. albicans,* since this pathogen is a known to be polymorphic yeast.

The cell wall of *C. albicans* (Fig. 1.2a and b) accounts for about 20 % of the total cellular dry weight and plays an essential role in virulence, adhesion and biofilm formation (Díaz-

Jiménez et al. 2012; Klis et al. 2009) (to be discussed in more detail later). In the late 1970's, transmission electron microscopy (TEM) revealed that the cell wall of *C. albicans* is a multi-layered structure (Poulain et al. 1977) and during the following decades, the cell wall of this yeast became the most extensively studied cell wall of any potentially pathogenic fungus. Considering the cell wall of *C. albicans* in its unicellular growth phase, two main layers can be differentiated: the outer layer that is comprised of glycoproteins and an inner layer that contains skeletal polysaccharides (Fig. 1.2b; Gow et al. 2011). Generally, chitin which is a structural polysaccharide of the inner cell wall, comprises only ca. 2% (w/w) of the cell wall of *C. albicans* in its unicellular growth phase, and together with β-1,3-glucan (ca. 40%, w/w) constitute the inner layer of the yeast's cell wall and provides strength and cell shape (Gow et al. 2011; Ruiz-Herrera et al. 2006). The outer layer comprises of β-1,6-glucan, and mannans covalently bound to proteins on the cell surface, each carbohydrate comprising ca. 20% (w/w) and ca. 40% (w/w) of the total cell wall, respectively. Mannans in the outer cell wall are less structured and show low permeability and porosity, thereby protecting the cell against antifungal agents (Gow and Hube 2012).

The mannan layer in the yeast cell wall is formed by mannoproteins that are extensively glycosylated in the lumen of the rough endoplasmic reticulum (ER), as well as in the Golgi apparatus, before being transported to the cell wall (Cutler 2001). This glycosylation pathway has been well studied in *S. cerevisiae* with multiple protein complexes found to be responsible for the successive elongation of the poly-α1,6-mannan backbone (Orlean 2012). A few studies exist to compare whether the same gene families also play a role in the glycosylation of *C. albicans* cell wall mannoproteins (Bai et al. 2006; Bates et al. 2006; Cutler 2001; Hall et al. 2013; Southard et al. 1999). The mannan layer of *C. albicans* was found to be held together by α-1,6, α-1,3, α-1,2 and β-1,2 linkages, as well as phosphodiester bonds (Nelson et al. 1991). The cell wall mannans containing N-linked oligomannosides, each comprising of a backbone of α-1,6-linked mannose residues, are linked to cell wall proteins via N-glycosidic bonds to asparagine residues (Fig. 1.2a). In both *S. cerevisiae* and *C. albicans,* this core structure of the N-glycan is formed through the activity of Och1 (α-1,6-mannose transferase) (Bates et al. 2006; Orlean 2012). Attached to the mannan backbone of *C. albicans* are either single mannose residues or oligomers containing variable numbers of mannose residues joined via α-1,2 and terminal β-1,2 linkages with occasional terminal α-1,3 linkages. In *S. cerevisiae,* the mannan polymerase I complex (M-pol 1, compose of Mnn9 and Van1 α1,6-mannosyltransferases) adds the first α-1,6-mannose residues to the core structure,

after which the mannan polymerase II complex (M-Pol 11, composed of Anp1, Mnn9, Mnn10, Mnn11 and Hoc1 α1,6-mannosyltransferases) elongates the α-1,6 mannan backbone, while enzymes belonging to the families *MNN2* and *MNN5* further modifies the backbone with addition of more mannose residues (Orlean 2012). Orthologues for Mnn9, Mnn2 and Mnn5 have been found in *C. albicans* (Bai et al. 2006; Hall et al. 2013; Southard et al. 1999). In both *S. cerevisiae* and *C. albicans* the terminal α-1,3-linked mannose are catalysed by representatives of the family *MNN1* (Bates et al. 2013; Orlean 2012). Mannose residues with β-1,2 linkages, present in the cell wall of *C. albicans* and absent in that of *S. cerevisiae,* are added via the action of β-1,2-mannosyltransferases (Courjol et al. 2015). Mannan can also be attached to cell wall proteins by O-glycosidic bonds to threonine or serine residues (Nelson et al. 1991). These O-linked unbranched oligomannosides usually consist of two to six mannose residues joined *via* α-1,2 linkages, and can be capped with either α-1,3 or β-1,2-mannose linked residues. It is estimated that a large amount of mannoproteins in the cell wall of *C. albicans* is O-mannosylated rather than N-mannosylated (Strahl-Bolsinger et al. 1999).

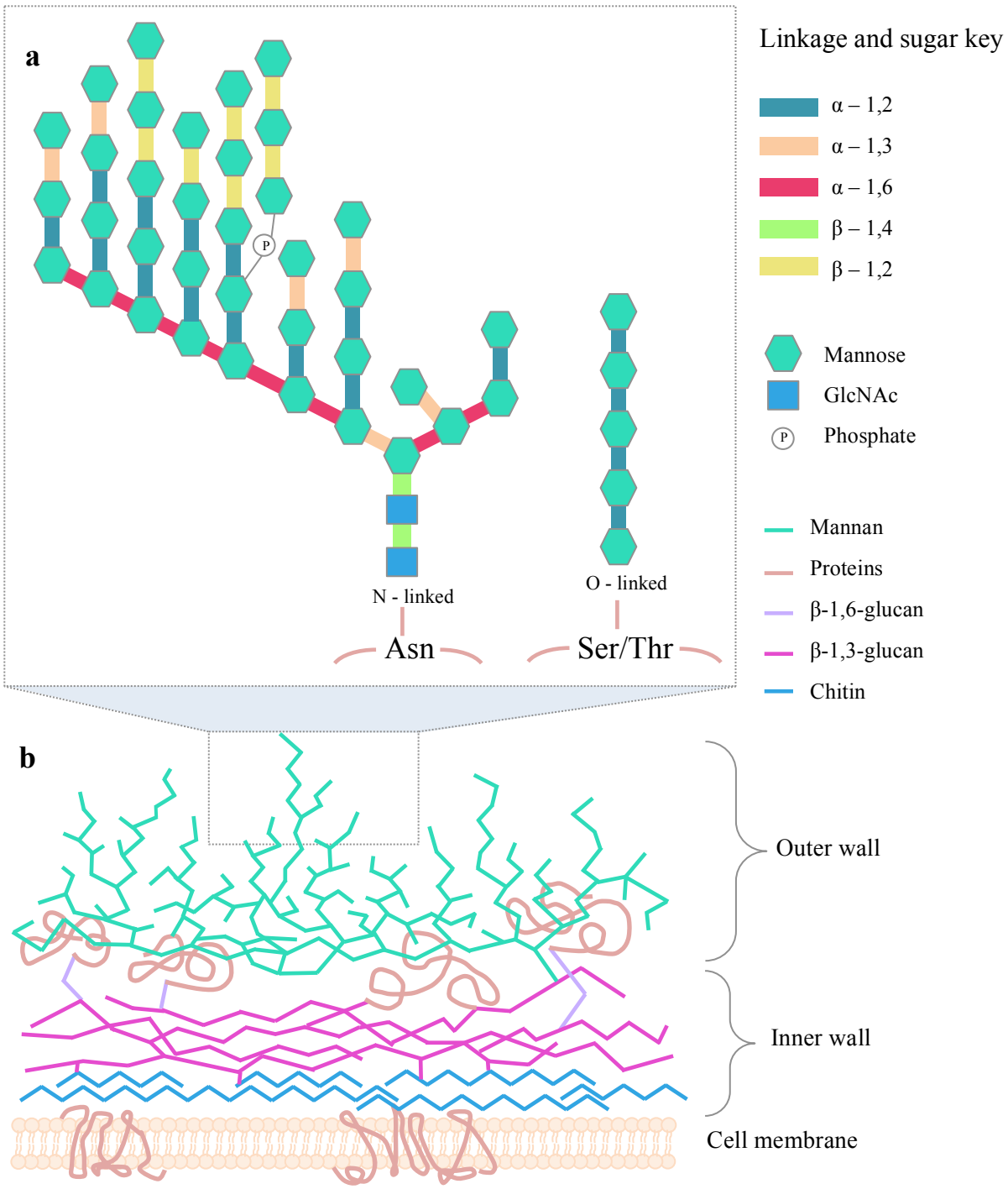


Fig. 1.2 Schematic representation of the cell wall of *C. albicans.* **a** The mannans on the outer layer can either be attached to the cell wall proteins by N-glycosidic bonds to asparagine residues or be attached by O-glycosidic bonds to threonine or serine residues. The N-linked mannans consist of a α-1,6 backbone to which either single mannose residues or oligomers are joined via α-1,2 linkages with terminal β-1,2 or α-1,3 linkages. **b** The cell wall of *Candida albicans* is characterized by two distinct layers. The inner layer containing β-1,3-glucan (ca.

40%) and chitin (ca. 2%), while the outer layer contains β-1,6-glucan (ca. 20%) as well as mannans covalently bound to proteins (ca. 40%) on the cell surface (Gow et al. 2011; Ruiz-Herrera et al. 2006).

The mannan and chitin containing layers of yeast cell walls can readily be visualised using simple staining methods in combination with fluorescence microscopy. Usually, Calcofluor white and fluorescent concanavalin A is used to stain the chitin and mannan layers, respectively (Rueda et al. 2014; Travassos et al. 1977). Figure 1.3 shows the outer mannan layer and a clearly visible chitin layer in the cell wall of *C. albicans*. It must be noted however, that the relative quantities of each of these cell wall components may vary due to a number of influencing factors, including morphogenesis (Hall 2015). The unicellular yeast form of *C. albicans* contains 3-5 times less chitin than its hyphal form (Braun and Calderone 1978; Chattaway and Holmes 1968; Gow et al. 2011) and the chitin content of a yeast bud scar is much higher when compared to the remainder of the yeast cell (Cabib and Bowers 1971; Fig. 1.3). Alternatively, hyphal cells contain threefold more glucan and almost half the amount of mannan compared to unicellular yeast cells (Machová et al. 2015; Staniszewska et al. 2013). Interestingly, TEM images indicated that the mannan fibrils around a yeast cell are longer and more densely packed compared to the mannan around hyphal cells (Cheng et al. 2011).

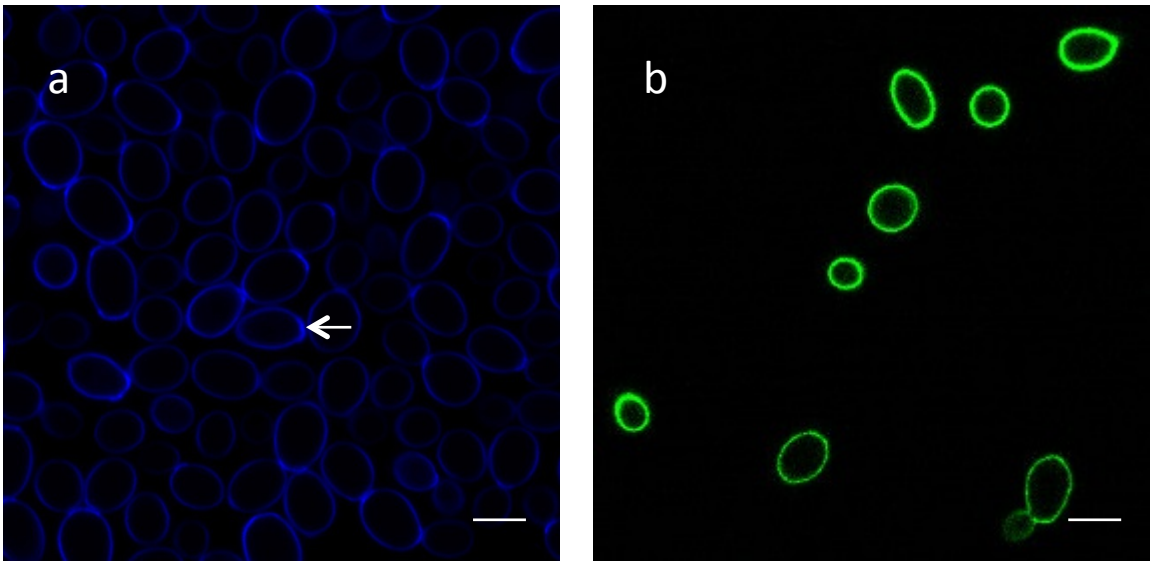


Fig. 1.3 Confocal images of calcofluor white and concanavalin A, fluorescein conjugate stained *C. albicans* CAB 1084 cells. **a** calcofluor white stained cells grown aerobically for 72 h at 26ºC in Luria Bertani (LB) broth. **b** concanavalin A, fluorescein conjugate stained cells grown aerobically for 72 h at 26ºC in LB broth. (The white arrow points to a yeast bud scar

containing more chitin than compared to the rest of that yeast cell.) Fluorescence microscopy conducted with a Carl Zeiss Confocal LSM 780 Elyra S1 and SR-SIM superresolution platform was used to capture cellular images. Scale bar represent 5µm.

1.2.3 Phenotypic switching and mating

In addition to the different morphological cell types found in *C. albicans,* (Fig. 1.1) the unicellular yeast phase can exist as different phenotypes (Table 1.1). It is known that this yeast can undergo phenotypic switching from ordinarily white-phase cells to opaque-phase cells. This was first discovered by Slutsky and co-workers (1987) and the strain, *C. albicans* WO-1, is being used in most studies to investigate the opaque properties of this yeast. While white-phase cells are spheroidal to ovoid and form white domed-shaped colonies, opaque cells are larger, ellipsoidal and their colonies appear darker and grow rather flattened on agar (Table 1.1). Both the mechanism and the stimuli involved in initiating the switch between the white and opaque cell forms are not fully understood. Yet, it seems that an initial incubation of two days at 25°C under anaerobic conditions, prior to aerobic incubation at 25°C, will initiate the switch (Ramírez-Zavala et al. 2008). Typically, this switch can be induced on Lee's medium (Lee et al. 1975), which may be supplemented with 5 µg ml^{-1} phoxine B to selectively stain the opaque colonies pink. Opaque cells are twice the size of white cells and have a pimple-like cell surface which can be examined using scanning electron microscopy (SEM; (Anderson et al. 1990). It is thought that these "pimples" contribute to pheromone signalling between mating pairs of *C. albicans* cells and it may be that they provide the first sites of membrane fusion during mating (Miller and Johnson 2002).

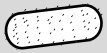
Even though the presence of haplo- and diplophases in *C. albicans* were already recognized nearly 50 years ago (van der Walt, 1970), mating via a parasexual cycle was only demonstrated about a decade ago (Dumitru et al, 2007; Forche et al. 2008). For effective mating, *C. albicans* has to undergo phenotypic switching. Miller and Johnson (2002) showed that opaque cells mate approximately 10^6 times more efficiently than white cells. It was thought that mating occurs better at lower temperatures, mostly at 25°C, but then Dumitru et al. (2007) demonstrated that *C. albicans* can also mate at 37°C whilst under anaerobic conditions. They showed that opaque cells remain stable for at least one week at 37°C under anaerobic conditions, but switch back to the white form within an hour at 37°C under aerobic

conditions. Therefore, it seems that anaerobic conditions are ideal for mating, suggesting that it may occur in the anaerobic parts of the human GI tract. It was suggested that the ability to mate under anaerobic conditions at 37°C may contribute to the survival of this yeast as commensal within its human host (Dumitru et al. 2007).

At first it was believed that only *C. albicans* strains homozygous at the mating type locus (*MTL*, **a**/**a** or α/α), were capable of switching to the opaque form (Miller and Johnson 2002). However, Xie et al. (2013) demonstrated that a number of **a**/α strains were also capable of switching under certain conditions. These opaque cells exhibited similar phenotypes of typical *MTL* homozygous opaque cells, although they lacked the ability to mate. In addition, Pande et al. (2013) demonstrated that heterozygous *C. albicans* strains could switch within the mammalian gastrointestinal (GI) tract, but could sometimes remain in the white form during *in vitro* experimentation. They showed that these GUT (gastrointestinally induced transition) cells (Table 1.1) are morphologically and functionally different than opaque cells and they express a transcriptome that is optimized for the digestive tract. GUT cells were also found to outcompete wild-type cells in mixed infections. It was suggested that GUT cells are a commensal cell type since virulence-associated genes are downregulated, resulting that the pathogen exists in a benign state within the gut. Yet, these GUT cells are also able to switch and turn pathogenic when the immune system becomes compromised. It is tempting to ask, whether the primary niche of this organism is truly the human host, and why it would switch to a specific phenotype to favour its survival within this host?

A fourth phenotype of *C. albicans* was recently discovered, the so-called "gray" phenotype (Table 1.1) which differs from the white, opaque and GUT cells in several aspects (Tao et al. 2014). While GUT cells are more or less the same size and shape as opaque cells, but lacking the cell wall pimples (Pande et al. 2013), the gray cells are notably smaller in size than both the opaque and GUT cells. The gray cells are stable in a variety of laboratory conditions, while the GUT phenotype is only observed when the cells move through an animal gut. Furthermore, gray cells show higher secreted aspartyl proteinase (Sap) activity than opaque and white cells, while this virulence gene is downregulated in GUT cells (Tao et al. 2014). From the above, it is evident that the yeast *C. albicans* has remarkably adapted to survive under different environmental conditions within the human body, where it may exist either as a commensal or a pathogen (Moran et al. 2012).

1 **Table 1.1** Different phenotypes to which the yeast *C. albicans* may switch, depending on environmental conditions.

Characteristics	**Phenotype**				**Reference**
	White	**Opaque**	**GUT**	**Gray**	
Cellular appearance	Round and small 	Elongated and large 	Elongated and large 	Elongated and small 	Anderson et al. 1990; Pande et al. 2013; Tao et al. 2014
Cell size (volume µm^2)	85-135	170-290	170-290	30-55	Pande et al. 2013; Tao et al. 2014
Colony appearance on solid media	White, domed and shiny	Opaque and flattened	Dark and flattened	Gray and shiny	Pande et al. 2013; Tao et al. 2014; Slutsky et al. 1987
Perceived niche	Commensal and bloodstream	Skin	Commensal in gastrointestinal tract	Skin	Gow et al. 2002; Kvaal et al. 1999; Pande et al. 2013; Tao et al. 2014
***MTL* configuration**	Heterozygous or homozygous	Usually homozygous, but can be heterozygous	Heterozygous	Heterozygous or homozygous	Miller and Johnson 2002; Pande et al. 2013; Tao et al. 2014; Xie et al. 2013
Mating competency	Low	High	Intermediate	Intermediate	Miller and Johnson 2002; Tao et al. 2014

1.3 Commensal and Pathogen

1.3.1 Commensal

It has been known for some time that *C. albicans* can live as a relatively harmless commensal within many anatomical sites of humans and other warm blooded animals (Bougnoux et al. 2006; Clayton and Noble 1966; Sobel 2007). The yeast can colonize niches, such as the GI and genitourinary tracts, oral cavities and skin of healthy individuals, and under normal circumstances will not cause significant infections (Chester and Cooper 2011). It is widely accepted that the GI tract is the main reservoir for *C. albicans* (Nucci and Anaissie 2001) and that most systemic infections originate from here (Voss et al. 1994). Interestingly, although it is known that *C. albicans* is a well-established commensal within healthy humans, few studies were aimed at elucidating the commensal lifestyle of this yeast. This can be ascribed to the difficulty in finding *in vivo* models representing the GI tracts of humans (Wilson et al. 2012). Since *C. albicans* does not colonize the GI tracts of rodent models, some form of treatment, such as antibiotics is needed to establish colonization by the yeast. Despite this, some *in vivo* studies have been conducted attempting to expand the available knowledge of the commensal lifestyle of *C. albicans* (Chen et al. 2011; Pande et al. 2013; Pierce and Kumamoto 2012; White et al. 2007). In contrast to the relatively limited information on the commensal lifestyle of *C. albicans*, extensive data is available on the pathogenicity of this yeast.

1.3.2 Transition to pathogen

The mechanisms allowing *C. albicans* to break through the intestinal barriers and cause infection are known to be complex (Reviewed in Yan et al. 2013). These barriers, which include mechanical-, immune-, microbial- and chemical barriers, form a multifaceted network of commensal microbes, secretion factors and enzymes, as well as cells and signalling receptors that jointly protect the host from enteric infections (Fig. 1.4). The communication within this network is critical in maintaining a well-organized immune system (Gill et al. 2011). If one or more components are compromised, it can cause a cascade of events resulting in negative consequences for the host. Since it is believed that the GI tract is the main reservoir for *C. albicans* (Nucci and Anaissie 2001), this niche's barriers will be discussed briefly in the next section.

1.3.2.1 Mechanical barrier. The intestinal mechanical barrier is one of the most important lines of defence for the host (Yan et al. 2013) (Fig. 1.4). This barrier includes a mucus layer, the mucosal lamina propria, epithelial cells joined by tight junctions between these cells. In the intestines, there are four major types of epithelial cells (Natividad and Verdu 2013): the absorptive enterocytes, which make up more than 80% of all epithelial cells; the hormone producing enteroendocrine cells; the mucous producing goblet cells; and the antimicrobial and growth factor producing Paneth cells. The intestinal enterocyte and goblet cells secrete mucin glycoproteins forming the elastic mucus layer, which is the first barrier *C. albicans* needs to overcome to initiate colonization of the GI tract lining (Yan et al. 2013). Before *C. albicans* can invade epithelial cells and cause damage, it needs to bind to this mucus layer, by first binding to the glycopeptides of mucin and thereafter invasion of the mucin is facilitated by selective expression of *SAP* (Secreted aspartyl proteinase) genes (Naglik et al. 2003). The invasion of the pathogen can also occur when epithelial cells are damaged due to illness, for example during gastrointestinal disease, where after *C. albicans* may infect the host (Coates 2005). It must be noted however, that the mucosal response toward microorganisms is not only important during infection, but is also responsible for homeostasis in the host and its microbiome (Bevins and Salzman 2011).

1.3.2.2 Immune barrier. This specialized barrier against enteric infection (Fig. 1.4) is known to be complex (Yan et al. 2013). The GI tract's immune system, known as the gut-associated lymphoid tissue (GALT) is where initial immune responses are induced. It primarily consists of Peyer's patches and mesenteric lymph nodes and protects the GI tract from the stomach to the colon (Czerkinsky et al. 1999; Luongo et al. 2009). The M (microfold) cells which differ from the other epithelial cells due to its lack in microvilli, overlay the Peyer's patches and allow for antigens and microorganisms to be in direct contact with the immune system to ensure specialized immune responses (Kucharzik et al. 2000). The intestinal lamina propia, which is the diffuse effector site of the GALT, contains antigen-presenting cells, including dendritic cells, macrophages as well as T cell subsets (mainly CD4+ T helper cells). Additionally, the site includes B cells and plasma cells and produces immunoglobulin A (IgA). Interestingly, it was recently discovered that *C. albicans* is able to use M cells as a vehicle to cross the intestinal barrier (Albac et al. 2016). However, the immune system will recognise this pathogen and enable a cascade of events to prevent *C. albicans* from infecting host tissue.

The pattern recognition receptors (PRRs) on the surfaces of cells from the innate immune system recognise conserved structures called pathogen associated molecular patterns (PAMPs), of microorganisms (van de Veerdonk et al. 2012). There are three major families of PRRs that can recognise *C. albicans*: the C-type lectin receptors (CLRs), toll-like receptors (TLRs) and the nucleotide oligomerization domain-like receptors (NODs) (van de Veerdonk et al. 2012). All of the main cell wall carbohydrate components of fungal walls serve as PRRs. Mannans, which form part of the outer layer of *C. albicans* cell wall (Fig. 1.2), are recognised by a variety of PRRs. These include the C-type lectin macrophage mannose receptor (MMR), the TLRs (TLR2 and 4) as well as galectin 3. The N-linked glycans on the cell wall are recognised by the MMR (Yamamoto et al. 1997; Netea et al. 2006), while the O-linked glycans are recognised by TLR4 (Netea et al. 2002; Netea et al. 2006). In addition, the glycolipid phospholipomannan is recognised by TLR2 (Jouault et al. 2003) and the galectin-3 receptor plays a role in recognising the β-1,2 mannose residues (Jouault et al. 2006). The β-1,3 glucan is recognised by dectin-1 and TLR2 (Brown and Gordon 2001; Gow et al. 2007; Netea et al. 2006). Lastly, chitin is also involved in the immune recognition of *C. albicans* by blocking dectin-1-mediated engagement with fungal cell walls (Mora-Montes et al. 2011). When *C. albicans* triggers these receptors, it results in the activation of cells from the first line of the innate host defence. *Candida albicans* thus needs to bypass these cellular responses, as well as other barriers, before it can invade host tissue and cause infection.

1.3.2.3 Microbial barrier. External and internal surfaces of the human body harbour complex indigenous microbial consortia that include both aerobic and anaerobic bacteria (Tancrede 1992). The bacterial microbiome of the GI tract comprises of more than 500 species (Fig. 1.4) while the cells in this biome outnumbers the cells of their human host by a factor of ten to one (Gravitz 2012). These bacteria belong to six different phyla, i.e. Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria and Proteobacteria. It was found that the proportion of each phylum differs at each anatomical site within the GI tract (Cho and Blaser 2012) and it is generally accepted that this vast and diverse mammalian microbiome protects the host against fungal and bacterial pathogens. Findings in this regard have already been published during the 1980s (Kennedy and Volz 1985).

The results obtained by Kennedy and Volz (1985) confirmed the role of the microbiome in controlling *C. albicans* mucosal colonization and dissemination into the bloodstream. Using a hamster model these authors demonstrated that the intestinal microbiome inhibits *C. albicans*

colonization and dissemination. They also proposed that this may happen via at least two mechanisms: (i) reducing the size of the *Candida* population and (ii) inhibiting the mucosal association of *Candida* cells. The first mechanism can be ascribed to bacterial antagonism and competition for limited nutrients. For example, lactic acid bacteria mainly control *C. albicans* growth in the lower parts of the female reproductive tract (Boris and Barbés 2000). Most *Lactobacillus* strains release hydrogen peroxide (H_2O_2), lactic acid or other organic acids that inhibit growth of *C. albicans.* These bacteria may also produce bacteriocin-like substances which can suppress yeast growth. The second mechanism proposed by Kennedy and Volz (1985), by which the microbiome protects the host from fungal infections is by forming a barrier between the epithelium cells and the pathogen. Bacteria form thick layers on the mucus gel covering the epithelium, thus preventing the yeast cells from penetrating into the mucus. Thus, not only does the microbiome compete with the pathogen for adhesion sites, but its members also secrete substances that may reduce the ability of *C. albicans* to attach to mucosal structures (Kennedy and Volz 1985). For example, certain bacteria can produce secondary bile acids, volatile fatty acids or biosurfactants that decrease pathogen binding by modifying *Candida* adhesins and/or mucosal receptors (Boris and Barbés 2000; Kennedy and Volz 1985).

1.3.2.4 Chemical barrier. The mammalian digestive tract secretes a number of compounds (Fig. 1.4) which contribute to the chemical control of *C. albicans* colonization (Yan et al. 2013). These compounds include various digestive enzymes, lysozyme, gastric acid, bile (acids and salts) and mucopolysaccharides. It was previously demonstrated that bile salts, one of the components of bile, exert an antifungal effect towards *C. albicans* (Marshall et al. 1987). These bile salts which may be derived from chenodeoxycholic acid, cholic acid, deoxycholic acid, glycocholic acid, glycodeoxycholic acid, hyodeoxycholic acid as well as lithocholic acid were found to be fungistatic rather than fungicidal. More recently, when the antifungal activity of the three major bile salts, i.e. those containing cholic acid, glycocholic acid and taurocholic acid was investigated, it was found that the salt of cholic acid had the strongest antifungal activity towards *C. albicans* (Kong et al. 2011).

In addition to bile salts, other antimicrobial compounds are secreted into the small intestine. It is known that Paneth cells for example, secrete antimicrobial proteins (AMP) such as α-defensins (Faderl et al. 2015). In must also be noted that the mucus layer not only serves as a physical barrier, but also acts as a biochemical wall, trapping digestive enzymes, as well as

the above-mentioned AMP and secreted IgA, thereby increasing the concentration of these antimicrobial compounds to the detriment of potential pathogens.

If one or more of the above-mentioned barriers are compromised, it may result in a cascade of events leading to infection of the host by *C. albicans* which may result in candidiasis and candidemia (Gill et al. 2011).

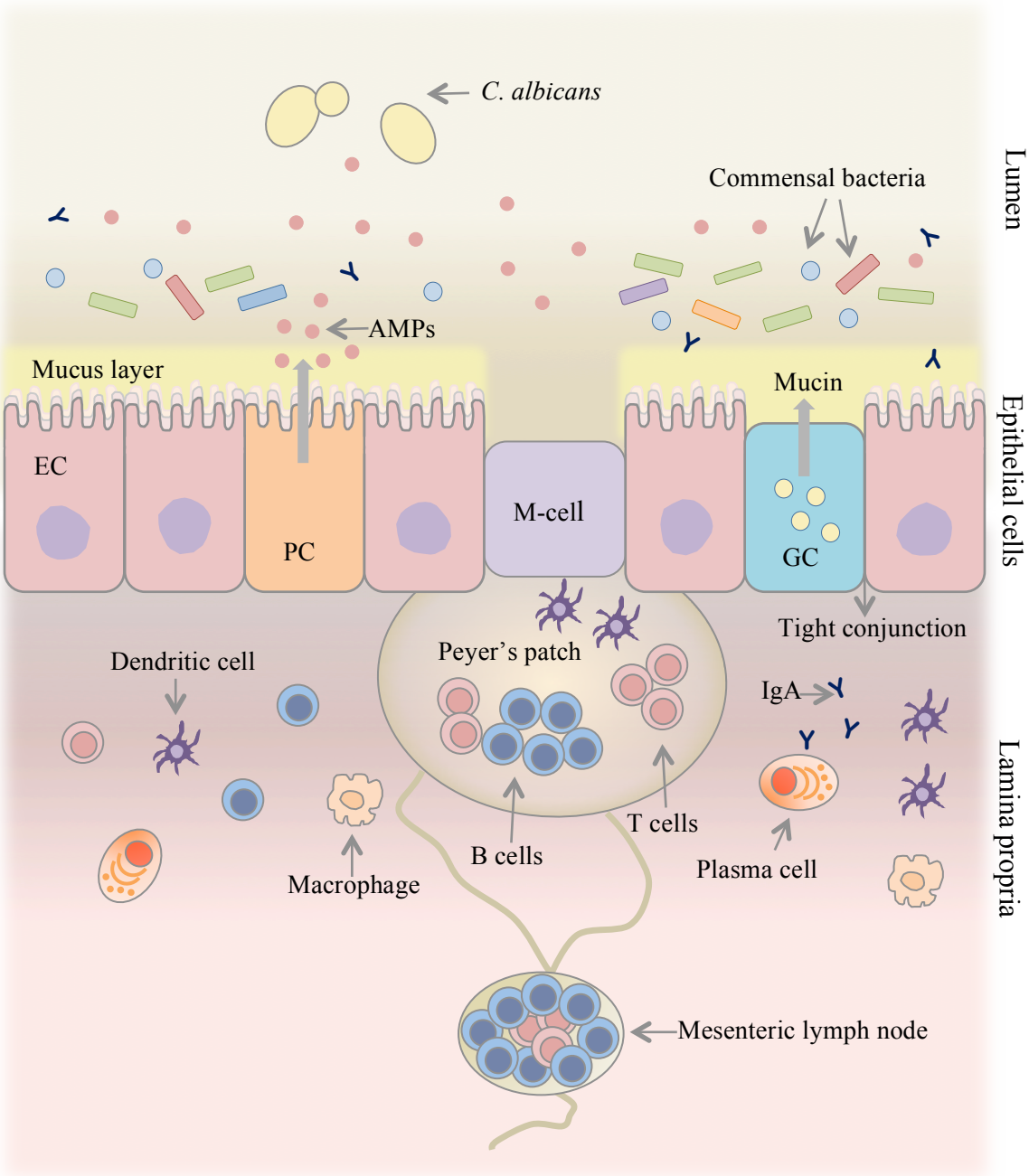


Fig. 1.4 Simplified representation of the intestinal barrier that *C. albicans* need to overcome in its human host to be able to switch from a commensal to a pathogen and cause infection (Adapted from Macpherson a Harris 2004 and Natividad and Verdu 2013). The mechanical barrier mainly includes the mucus layer, the mucosal lamina propria and the four different epithelial cells: enterocytes (EC), enteroendocrine, goblet (GC), and Paneth cells (PC). Peyer's patches, mesenteric lymph nodes and M cells play an important role in initial immune responses and contributes to the function of the chemical barrier. Commensal

bacteria which comprise of about 500 different species, protect the host by preventing pathogens to adhere to the epithelial cells and simultaneously inhibiting their growth.

1.4 Candidiasis and Candidemia

Of the roughly 150 *Candida* species that were described thus far, only 15 species are considered medically relevant (Moran et al. 2012). Furthermore, over 90% of *Candida* infections are caused by only five species, i.e., *C. albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis*; *C. albicans* is responsible for 50 - 60% of these cases. It is know that endogenous infections caused by this yeast are triggered by a compromised immune system, or when the competing microbiome is damaged. Vaginal and oral candidal infections are common even in mildly immunocompromised individuals, or when *C. albicans* outcompetes the microbiome after extensive antibacterial treatment (Calderone and Fonzi 2001). A superficial or mucocutaneous infection caused by *C. albicans*, is known as candidiasis.

Oral candidiasis is the most common opportunistic fungal infection among individuals infected with HIV/AIDS (Brawner and Hovan 1995). In some severe cases patients may die from advanced oesophageal colonization. Until now, *C. albicans* was found to be an important opportunistic pathogen, especially in patients with a compromised immune system, such as those subjected to cancer chemotherapy, implant or transplant procedures or suffering from HIV/AIDS. The occurrence of HIV/AIDS continues to increase globally, especially in developing countries of Africa. For example, Africa has the world's highest occurrence of HIV infections and according to the report on the global AIDS epidemic (UNAIDS, 2013), it is estimated that 24.7 million adults and children older than the age of 15 years, live with HIV/AIDS in sub-Saharan Africa alone. In South Africa, 5.96 million people suffer from HIV (according to the GERMS-SA Annual Report, 2015).

Other major syndromes caused by *C. albicans* are: dermatitis, cystitis, myositis, hepatic dysfunction and mental confusion, which may occur singly or in combination (Chester and Cooper 2011). In severe cases, *C. albicans* can penetrate deeper into host tissues, from where it may enter the bloodstream, a condition known as candidemia. Within the bloodstream it has the potential to invade virtually any body site and organ where it can cause life threatening systemic infections (Hube 2004). *Candida albicans* is one of the few microbial

pathogens that have the potential to cause infection at an extensive range of diverse environments within its host and is characterized by a number of features that enhances its virulence within the host (Calderone and Fonzi 2001).

1.5 Virulence Factors

For *C. albicans* to survive and proliferate in such diverse environments within its host, it has to be able to withstand significant changes in oxygen and carbon dioxide levels, pH, osmolarity, temperature and availability of nutrients. Kumamoto (2008) reviewed *C. albicans* niche-specific gene expression during infections and summarized how this yeast can overcome challenges, unique to certain sites, within the host tissue.

Testimony to the resilience of *C. albicans* is its ability to thrive over a remarkably broad range of environmental pH conditions. Within its host the yeast may encounter pH values ranging between 2 and 10 (Davis 2003). In the GI tract pH varies from very acidic in the stomach (pH 2), to less acidic in the duodenum (pH 5) to alkaline in the intestine (pH 7.7) (Bensen et al. 2004). The pH of the oral cavities varies between 5 and 7 (Scholz et al. 2008) due to changes in diet, the metabolism of other microorganisms and salivary flow (Kleinberg and Jenkins 1964). In the bloodstream *C. albicans* encounters a neutral pH of 7.4 and an acidic pH of 4.5 in the vaginal tract. Despite these differences in pH, *C. albicans* is known to thrive in all of the above sites in the human body (Calderone and Fonzi 2001); nevertheless, pH does have an effect on the plasma membrane, protein activity, maintenance of the proton gradient, and nutrient availability (Davis 2003). In addition, pH affects morphogenesis of *C. albicans* (Saporito-Irwin et al. 1995). Acidic conditions favour yeast formation and in alkaline environments *C. albicans* grows in the hyphal form (Sudbery et al. 2004). It is generally accepted that the ability of *C. albicans* to thrive and adapt to such a broad range of extracellular pH levels contributes to its pathogenicity. Davis (2003) summarized the gene expression and pathways responsible for detecting changes in extracellular pH and suggested that a better understanding of these pathways in *C. albicans* will yield insights into how this yeast is able to be so successful as a pathogen and commensal.

The ability of *C. albicans* to switch between different morphological states (Calderone and Fonzi 2001) is considered to be the most important virulence factor aiding the survival of *C. albicans* in its host (Biswas et al. 2007). However, contentions exist whether the yeast- or the

hyphal form is more virulent. Nevertheless, both forms seems to play a role in pathogenesis, and it is thought that the yeast form is important for dissemination of the pathogen through the bloodstream, while the hyphal form appears to be important in tissue invasion and is therefore often found at sites of invasion (Gow et al. 2002).

As mentioned previously in this review, the cell wall, which also determines the morphology of *C. albicans*, plays a pivotal role in virulence (Díaz-Jiménez et al. 2012). Adhesins expressed on the cell surface of *C. albicans*, allow the yeast to adhere to the different sites within its host (Moyes et al. 2015). Although it is thought that the hyphae are the more adherent form of this yeast, initial adhesion to human epithelial cells are mostly conducted by the yeast cells. The most studied group of *C. albicans* adhesins, is the agglutinin-like sequence (Als) proteins (Mayer et al. 2013). Three Als (Als2, Als3 and Als4) in *C. albicans* were identified as the most important proteins contributing to adhesion of this pathogen to human epithelial cells (Zhao et al. 2004; Zhao et al. 2005). A few other adhesins also contribute to the attachment of this yeast to various substrates (Bates et al. 2007; Li and Palecek 2003; Li and Palecek 2008; Sandini et al. 2007; Zhu and Filler 2010), but will not be discussed in this review. The above-mentioned ability of *C. albicans* to adhere to surfaces renders it difficult to eradicate this opportunistic pathogen from its host.

Another cell wall related virulence factor, is the phospholipomannan (PLM) occurring in the outer layer of the cell wall (Fradin et al. 2015). It's been demonstrated that this phylogenetically unique molecule, which consist of a glycan moiety composed of β-1,2-oligomannoasides can act as an adhesin (Fradin et al. 1996; Fradin et al. 2000); as well as stimulate macrophages apoptosis (Devillers et al. 2013; Ibata-Ombetta et al. 2003). Interestingly, due to the presence of β-1,2-oligomannoasides in the *C. albicans* mannan layer, a glycan moiety not expressed in *S. cerevisiae*, galectin-3 (a PRR of the human host) is able to discriminate between these two yeast species (Kohatsu et al. 2006).

Mannosylation in *C. albicans* also influences this pathogen's virulence profile (reviewed by Hall and Gow 2013). Since mannosylation mutants of both the O-linked and N-linked mannans express an altered mannan structure, while the cells are still viable, these mutants can be used as tools to explore certain traits. It was found that all mutants involving in O-mannan showed attenuation of virulence in murine systemic infection models (Buurman et al. 1998; Timpel et al. 1998; Timpel et al. 2000; Munro et al. 2005; Rouabhia et al. 2005). Most

of these mutants showed adhesion defect, such as a reduced ability to adhere to human epithelial cells. Other traits included, reduced capacity to form biofilms, increased sensitivity towards cell wall perturbing agents and reduced cell wall integrity. Similarly, N-mannan mutants showed to be less virulent compared to wild-type strains. The *mnn5* mutant strains were found to be less virulent; however the reason for this was unclear (Bai et al. 2006). Later, *mnn2* mutant strains were also found to show attenuated virulence (Hall et al. 2013). It was proposed that loss of the α-1,2-mannan in such mutants would result in reduced immune recognition of *C. albicans* by the peripheral blood monocytes. Researchers recently confirmed how these mutants may contribute to *C. albicans* being less virulent, by studying *mnn10* mutants (Zhang et al. 2016). They demonstrated that *mnn10* mutants of *C. albicans* are also less virulent compared to its wild-type. Firstly, they identified a novel α-1,6-mannosyltransferase encoded by *MNN10* in *C. albicans* and confirmed that Mmn10 is required for the extension of the mannan backbone. They then demonstrated that inhibition of α-1,6-mannose backbone extension induces enhanced host innate immune response by unmasking the concealed β-(1,3)-glucan and exposing it to dectin-1. It therefore seems that an important virulent function of the mannans in the outer layer of the yeast call wall is to mask the inner cell wall components, containing highly immunostimulatory moieties, from the host's immune system.

The ability of *C. albicans* to form biofilms further advances the resilience of *C. albicans* under challenging environmental conditions (Kumamoto and Vinces 2005) and contributes to the virulence of this yeast (Seneviratne et al. 2008). A biofilm is defined as an aggregate of microorganisms in which cells adhere to one another on a surface, embedded within a matrix of extracellular polymeric substances. Microorganisms undergo a change to a sessile lifestyle when the first cells start to attach to a surface. Cells within a biofilm usually display phenotypic features that vary from the planktonic or free-floating cells (Costerton et al. 1995). In addition, it's been demonstrated that the yeast cells that disperse from the mature biofilm are more virulent compared to the planktonic cells (Uppuluri et al. 2010). Dental plaque is a well-known example of a natural biofilm formed by bacteria. *Candida albicans* however, can also form a biofilm on dental enamel as well as on almost all medical devices, such as stents, implants and catheters (Lamfon et al. 2003; Ramage et al. 2006). Such candidal biofilms are considered to pose serious nosocomial problems, especially since yeast cells within these biofilms are known to be more resistant, compared to free flowing cells, to antifungals (Hawser and Douglas 1995) and protect the yeast against antifungals. In the first

study where increased antifungal resistance was demonstrated, the authors found that various antifungal agents, including amphotericin B, fluconazole, itraconazole, and ketoconazole showed much less activity against *Candida* biofilms than planktonic cells (Hawser and Douglas 1995). A number of factors were identified that may contribute to this increased resistance of biofilm associated yeast cells. Firstly, these cells were found to show reduced growth rate compared to planktonic cells (Baillie and Douglas 1998). Secondly, the presence of a subpopulation of highly tolerant cells i.e. persister cells (LaFleur et al. 2006) and thirdly, the upregulation of drug efflux pumps (Ramage et al. 2002). Fourthly, the presence of an impermeable extracellular matrix shields the yeast against antifungals (Nett et al. 2007). In addition, biofilms may also provide an anaerobic environment thereby creating ideal conditions for potential mating (Dumitru et al. 2007). As a result of the above-mentioned increased resistance of biofilm associated yeasts medical devices, contaminated with candidal biofilms, are mostly removed as a result of ineffective antifungal treatment (Seneviratne et al. 2008). Surgical removal of these contaminated devices however, always poses a risk to the patient.

Another attribute to virulence, is the ability of *C. albicans* to produce hydrolytic enzymes (Mayer et al. 2013). Secreted aspartic proteases (Saps) contribute to the ability of this pathogen to penetrate and anchor it to host epithelial cells (Moyes et al. 2015). The proteases Sap 9 and Sap 10 were found to be crucial for the candidial infection process, since these enzymes were found to play a key role in adhesion of the pathogen to host epithelial cells (Albrecht et al. 2006). Phospholipases, another group of hydrolytic enzymes, which role in penetration is still unclear, seems to contribute to virulence by degrading phospholipids in the host's cell membranes (Ghannoum 2000). Similarly, the role in penetration of other candidal hydrolytic enzymes such as lipases, is also uncertain. However, a lipase mutant strain was found to show reduced virulence in murine intravenous infection model compared to the wild type strain (Gácser et al. 2007)

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1.6 Controlling *Candida albicans* growth

1.6.1 Antifungals used in pharmaceutics

Developing antifungal agents to treat fungal infections, including those caused by *Candida* strains, is quite challenging since fungal cells are metabolically similar to mammalian cells

(Ostrosky-Zeichner et al. 2010). With the exception of the fungal cell wall, there are limited potential pathogen-specific cellular targets for developing new antifungal drugs. Caspofungin was the first approved antifungal agent from a new class of antifungals, the echinocandins, identified in the new millennium (Kartsonis et al. 2003). Echinocandins inhibit synthesis of *β*-(1,3)-D-glucan, an essential component of many fungal cell walls including that of *C. albicans* (Fig. 1.2). Thus, this class of antifungal agent was the first to target a specific component of the fungal cell which is not present in mammalian cells (Denning 2003; Odds et al. 2003)

Apart from the cell wall, other antifungals target sterol-, DNA- and protein synthesis (Ghannoum and Rice 1999; Odds et al. 2003). Antifungal classes which affect fungal sterols are more abundant and are frequently used to treat life threatening mycoses (Odds et al. 2003). The antifungal polyenes, azoles and allylamines target the yeast cell membrane, more specifically, ergosterol biosynthesis. For example, the polyene amphotericin B, binds to ergosterol and cause leakage of the cytoplasm from the cell which eventually leads to cell death (Baginski et al. 1997). Amphotericin B is therefore viewed as a fungicidal agent (Graybill et al. 1997). On the other hand, the azole fluconazole, which inhibits 14α-demethylation of lanosterol in the ergosterol biosynthetic pathway (Bosschef 1995), only inhibit cell growth and is therefore deemed to be fungistatic (Graybill et al. 1997). Experimental evidence for the fungistatic and fungical properties of fluconazole and Amphotericin B respectively, is presented in Fig. 1.5.

Generally, most *C. albicans* isolates are enterily susceptible to all major classes of antifungal agents, including the azoles, echinocandins and polyenes (Moran et al. 2012). There are two antifungal susceptibility testing protocols generally used for antifungal testing, the broth dilution antifungal susceptibility testing of yeasts (NCCLS) and the Etest® (AB Biodisk, Solna, Sweden) method. Both methods include testing a concentration range of a particular antifungal agent against fungal strains to obtain the minimum inhibitory concentration (MIC) of the agent that is needed to either kill or inhibit a specific strain. Usually, the MIC is determined by visually observing cell suspensions in either microtiter or agar plates (depending on the protocol used) for growth after 24 and 48 hours of incubation. A rapid alternative method based on live dead staining in combination with flow cytometry (Pore 1994) have also been proposed, which can be used to obtain the MIC within a few hours compared to 48 h. The results of a number of studies on the effect of different antifungals

against *Candida* species indicated that the MIC obtained with the flow cytometry method is comparable to that obtained with the NCCLS and Etest methods (Joung et al. 2007; Ramani et al. 1997; Ramani and Chaturvedi 2000; Rudensky et al. 2005).

It must be noted that antifungal susceptibility testing protocols require aerobic testing of the antifungal agents and therefore MIC values for *C. albicans* under anaerobic conditions are not readily available. The only study to our knowledge where *C. albicans* was anaerobically tested against antifungals was done by Dumitru et al. (2004). They demonstrated that anaerobically grown *C. albicans* cells are much more resistant than aerobically grown cells to a series of antifungal agents. Interestingly, the authors suggested that the enhanced resistance of biofilm associated *C. albicans* may be due to the anaerobic interiors of such biofilms.

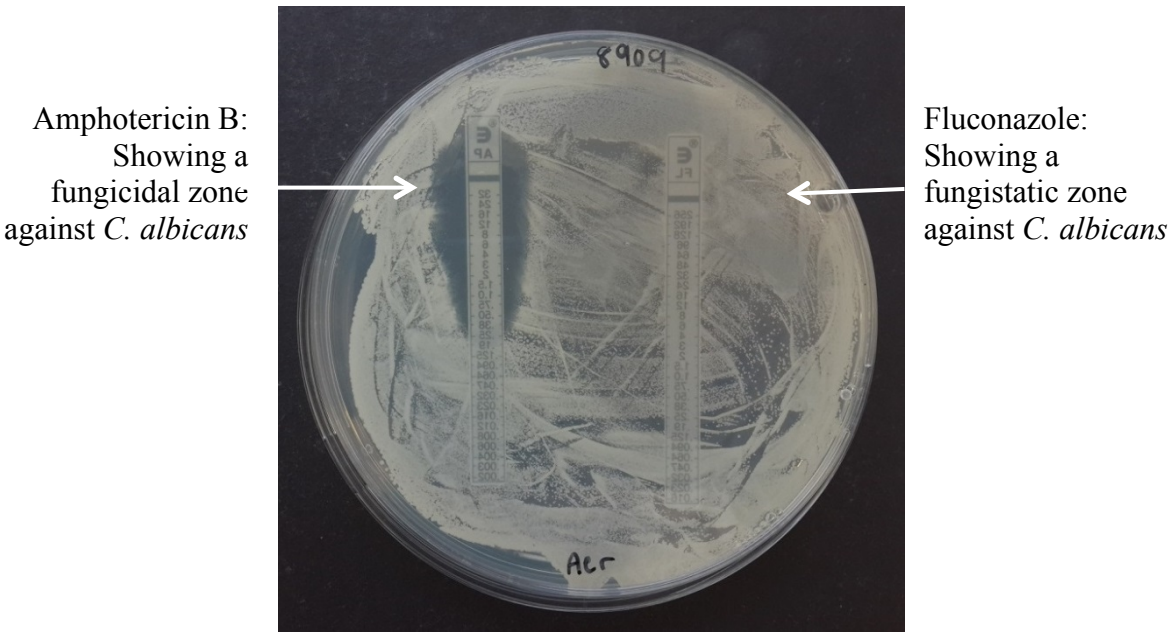

Fig. 1.5 The results of the so-called Etest (AB Biodisk, Solna, Sweden) showing different responses of a *C. albicans* strain towards a range of different concentrations of the fungicidal agent amphotericin B, and the fungistatic agent fluconazole.

1.6.2 Antimicrobials produced by bacterial pathogens of man

A few studies suggested, apart from the antagonistic effect of the human microbiome on *C. albicans*, that bacterial pathogens can also influence *C. albicans* growth (Peleg et al. 2010).

Experiments conducted in clinical microbiology trials, revealed that antimicrobials from certain proteobacterial strains including *Acinetobacter baumannii* (Peleg et al. 2008), *Burkholderia cepacia* (Boon et al. 2008), *Pseudomonas aeruginosa* (Hogan and Kolter 2002; Gibson et al. 2009), *Salmonella enterica* (Tampakakis et al. 2009) and *Xanthomonas campestris* (Wang et al. 2004), exert an antagonistic effect on *C. albicans*, more specifically, they all reduce the viability of *C. albicans* filaments.

A wide variety of bacteria are known to be mycolytic and were found to produce hydrolytic enzymes capable of degrading the different components of fungal cell walls, including chitinases, glucanases and proteases (Azam and Malfatti 2007; De Boer et al. 2005). Interestingly, previous research indicated that a combination of cell wall degrading enzymes and different antifungal compounds exert a synergistic antifungal effect on *Botrytis cinerea*, by inhibiting spore germination (Lorito et al. 1994). Also, others found that the antifungal compound prodigiosin and chitinase exert a synergistic antifungal effect on this fungus (Someya et al. 2001). Considering the relatively thick layer of mannans on the outside of this pathogen (Figs 1.2 and 1.3), bacterial chitinases may not be the principle enzymes that hydrolyze the cell walls of *C. albicans.* Even though it is known that some bacteria can produce α-mannanases (Cuskin et al. 2015; Gacto et al. 2000; Maruyama et al. 1994; Takegawa et al. 1989; Yamamoto and Nagasaki 1975), no studies focused on the synergistic effect of mannanase and antimicrobials on candidal growth. This phenomenon would especially be interesting to investigate under anaerobic conditions since all of the above mentioned experimentation were conducted under aerobic conditions.

1.6.3 Anaerobic growth

Unlike aerobic growth, anaerobic growth of *C. albicans* has been studied relatively little by microbiologists, even though the organism can be found in many anaerobic environments within its human host. These anaerobic environments include, the anaerobic parts of the GI tract (Hentges 1993) and oral sites, such as abscesses (McManners and Samaranayake 1990), infected root canals (Baumgartner et al., 2000), periodontal pockets (Reynaud et al. 2001), radicular dentinal walls (Siqueira et al. 2002) as well as sites of apical periodontitis (Waltimo et al. 1997). Interestingly, anaerobic growth may also be important for the survival and proliferation of *C. albicans* in the external environment, away from its mammalian host.

A well described external ecological niche for *C. albicans* is still lacking, but Stone et al. (2012) suggested that reducing, oxygen-limited zones of polluted wetlands and rivers may be a potential external reservoir for this pathogen. Using quantitative real-time PCR (qRT-PCR) to enumerate *C. albicans* from three different zones in a river over a period of eight months, they found that *C. albicans* was capable of sustainable growth in the reducing, oxygen limited zones. The reason and mechanisms allowing *C. albicans* to survive in the oxygen limited zones and not in the free flowing aerobic oxidizing zones, is still unclear.

As mentioned previously, bacterial antagonism may play a role in controlling this pathogen's growth. In an environment such as a polluted river, many bacterial species can be present which can potentially have a negative effect on *C. albicans* growth (Britz et al. 2013). It would be interesting to know whether the effect that bacteria have on *C. albicans* growth will differ under anaerobic conditions, especially in an external environment.

The possibility that *C. albicans* may exist in environmental niches, away from its mammalian host, is not generally considered during studies of the natural history of this yeast. Hube (2004) stated that it is surprising that *C. albicans* is exclusively associated with animals or humans and rarely found in environmental niches such as soil, since it has no definite nutrient requirements that would prevent it from surviving in the outside environment. This is in contrast to most other medically important fungi, including *Cryptococcus neoformans* (Casadevall et al. 2003), *Aspergillus fumigatus* (Latgé 2001) and *Histoplasma capsulatum* (Woods 2003), commonly found in external environments. Being a resident of the mammalian GI tract, *C. albicans* naturally occurs as a renowned potential pathogen in sewage sludge (Cook and Schlitzer 1981). However, a relatively low incidence of this yeast has been reported for sewage polluted waters (Marino et al. 1995). This has been ascribed to the die-off phenomenon of pathogens in polluted waters.

1.7 Conclusions

Over the decades many studies revealed that the yeast, *C. albicans*, is a polymorphic fungus. The cell wall of the unicellular yeast phase primarily consists of two layers, an inner layer containing the structural polysaccharide chitin and a low permeable relatively thick mannan containing layer known to protect the cell against antifungal agents. This mannan layer also plays a key role in virulence and protects the yeast cell from its surrounding environment.

Furthermore, *C. albicans* is known to be an adaptable opportunistic fungal pathogen that can survive and grow in many different niches on or in its mammalian host. Numerous studies have been conducted on the interactions of this yeast with its host, as well as its susceptibility to antifungal drugs such as amphotericin B and fluconazole. Very few studies however, addressed the ecology of *C. albicans* in the external environment away from an animal host. Even though this yeast is remarkably adaptable and able to switch between different morphological states to ensure survival, strains are only occasionally isolated from sewage sludge and waste waters contaminated with fecal material. However, recently indications showed that this pathogen is indeed capable of sustainable growth in anaerobic zones in a polluted river, while no evidence of such growth was obtained in the aerobic zones of the river. The reasons why *C. albicans* only survives in the oxygen limited zones of the polluted river, and not in the free flowing aerobic oxidizing zones, remain unclear. A number of studies showed that bacterial activity may impact the survival of *C. albicans*. Some research groups found that certain fungi, including *C. albicans* can be adversely affected by bacterial enzyme activity and secondary metabolites, such as prodigiosin. It must be noted however, that these studies were all performed under aerobic conditions. Bacteria/yeast interactions, as well as the effect of bacterial enzymes and secondary metabolites on *C. albicans* under anaerobic conditions has not commonly been studied, and therefore little is known about anaerobic or oxygen limited antifungal processes.

1.8 Project objectives

Keeping the above in mind, the overall aim of this project was to study the interactions between bacteria and *C. albicans* under both aerobic and anaerobic conditions, and to investigate how the candidial mannan layer contributes to these interactions.

The first objective (Chapter 2) was to study binary interactions between strains of *C. albicans* and selected bacteria originating from the same sewage polluted river environment, under both aerobic and anaerobic conditions. Also, we aimed to screen the bacteria for enzyme production and test whether, in combination with prodigiosin, mannan degrading enzymes exert greater synergistic antifungal activity against unicellular growth of *C. albicans* than chitinases

The second objective (Chapter 3) was to study binary interactions between strains of *C. albicans* and bacteria associated with the mammalian host, under both aerobic and anaerobic conditions; and to determine whether selected antifungal drugs in combination with mannan degrading enzymes exert synergistic antifungal activity against unicellular growth of *C. albicans* than the drugs alone.

The third objective (Chapter 4) was to use *C. albicans* and the model ascomycetous yeast *S. cerevisiae,* to study binary interactions between these yeasts and *Bacteroides* species under anaerobic conditions. Also, we aimed to evaluate the role of the yeast cell wall mannans in these interactions.

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Chapter 2

Binary Interactions of Antagonistic Bacteria with *Candida albicans* under Aerobic and Anaerobic Conditions

Published in Microbial ecology 71.3 (2016): 645-659.

Binary Interactions of Antagonistic Bacteria with *Candida albicans* under Aerobic and Anaerobic Conditions

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2.1 Introduction

The most common opportunistic fungal pathogen of man is the ascomycetous yeast, *Candida albicans* (Robin) Berkhout (1923) (Moran *et al*., 2012). Although this pleomorphic yeast is a well-known inhabitant of the human gastrointestinal and genitourinary tracts, it can become pathogenic in immunocompromised individuals (Wilson *et al*., 2012). Numerous studies implicated *C. albicans* as being responsible for the majority of candidiasis and candidemia cases around the globe (Guinea, 2014; Pappas *et al*., 2003; Pfaller and Diekema, 2007). However, in contrast to the multitude of authors studying the pathology of this yeast, very few studies addressed the ecology of *C. albicans* in the external environment away from an animal host.

Although few reports exist on the occurrence of *C. albicans* in the external environment, strains are occasionally isolated from sewage sludge and waste waters contaminated with fecal material (Buck, 1977; Cook and Schlitzer, 1981; Cooke *et al*., 1960). It therefore is contended that these environments are not the primary habitat of *C. albicans* and that the yeast's presence in them is rather a result of human contamination (Chester and Cooper, 2011). This relatively low incidence of *C. albicans* was ascribed to a highly adapted parasitic life cycle which resulted in the loss of physiological properties that may have allowed this

pathogen to survive and proliferate outside its human host. Using quantitative real-time PCR (qRT-PCR) however, we recently found indications that *C. albicans* is indeed capable of sustainable growth in the reducing, oxygen limited zones along the banks of a polluted river, while we obtained no evidence of such growth for this yeast in the more aerobic zones of the river (Stone *et al*., 2012). The reasons why *C. albicans* only survives in the oxygen limited zones of the polluted river, and not in the free flowing aerobic oxidizing zones, remain unclear. However, a number of studies showed that bacterial activity may impact the survival of fungi, including *C. albicans* (Bernhardt *et al*., 1995; De Boer *et al*., 2005; Kennedy *et al*., 1987).

A wide variety of bacteria are known to be mycolytic and produce hydrolytic enzymes capable of degrading the different components of fungal cell walls, including chitinases, glucanases and proteases (Azam and Malfatti , 2007; De Boer *et al*., 2005). A number of research groups found that fungi representing *Alternaria*, *Aspergillus, Botrytis, Fusarium*, and *Penicillium* were adversely affected by bacterial chitinolytic activity of *Aeromonas hydrophila*, *Enterobacter,* and *Serratia marcescens* (Brzezinska *et al*., 2014; Dahiya *et al*., 2005; Halder *et al*., 2013; Someya *et al*., 2001). Interestingly, strains representing these mycolytic proteobacterial species were previously isolated from the same nutrient rich sewage polluted river as *C. albicans* (Britz *et al*., 2013; Stone *et al*., 2012) and therefore they may have a negative impact on the survival of *C. albicans* within this river environment. In addition, it was found that almost all mycolytic bacteria that produce hydrolytic enzymes may also produce additional antimicrobials, and that this mixture of compounds may be crucial for degrading living hyphae (De Boer *et al*., 2005). Such antimicrobials may include secondary metabolites, like the red pigment prodigiosin produced by *S. marcescens* (Williamson *et al*., 2006), or toxin proteins, such as aerolysin produced by *A. hydrophila* (Howard and Buckley, 1982). It must be noted however, that the above mentioned studies were performed under aerobic conditions. The production of enzymes, proteins, and secondary metabolites under anaerobic conditions is not commonly studied, and therefore little is known about anaerobic or oxygen limited antifungal processes.

Studies that other researchers conducted on clinically relevant bacteria revealed that certain proteobacterial strains, including representatives of *Acinetobacter baumannii, Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Xanthomonas campestris,* may have an antagonistic effect on *C. albicans* by reducing the viability of its filaments (Boon *et*

al., 2008; Hogan and Kolter, 2002; Peleg *et al*., 2008; Tampakakis *et al*., 2009; Wang *et al*., 2004). While some of these studies evaluated the effect of bacterial antimicrobials on the yeast, others focussed on binary interactions within the substitute model host, *Caenorhabditis elegans,* at either 30ºC or 37ºC. However, in the absence of a host within a river environment, which may be notably cooler, complex interactions may occur between different bacteria and *C. albicans.* The enzymes of one bacterial species, together with antifungal compounds of another, may exert synergistic antifungal activity against the yeast. Interestingly, previous research indicated that prodigiosin and chitinase exert a synergistic antifungal effect on *Botrytis cinerea* by inhibiting spore germination (Someya *et al*., 2001). However, bacterial chitinases may not be the principle enzymes that hydrolyze the cell walls of *C. albicans* in nutrient rich sewage polluted river systems, because at cooler temperatures (i.e. 26ºC), *C. albicans* predominantly grows as a unicellular yeast (Lee *et al*., 1975; Shapiro *et al*., 2009) and contains less chitin than its filamentous form (Braun and Calderone *et al*., 1978; Chattaway *et al*., 1968; Gow *et al*., 2012).

Generally chitin comprises only ca. 2% (w/w) of the cell wall of *C. albicans* in its unicellular growth phase, and together with β-1,3-glucan (ca. 40%, w/w) constitute the inner layer of the yeast's cell wall (Gow *et al*., 2012, Ruiz-Herrera *et al*., 2006). The outer layer comprises of β-1,6-glucan, and mannans covalently bound to proteins on the cell surface, each carbohydrate comprising ca. 20% (w/w) and ca. 40% (w/w) of the total cell wall respectively. Interestingly, bacteria are capable of producing both β-mannanases (Dhawan and Kaur, 2007) and α-mannanases, the latter implicated in the degradation of mannans in fungal cell walls, including that of *C. albicans* (Cuskin *et al*., 2015; Gacto *et al*., 2000; Maruyama *et al*., 1994; Takegawa *et al*., 2009; Yamamoto and Nagasaki, 1975).

To explain the persistence and survival of *C. albicans* in the oxygen limited zones of rivers and wetlands, as was demonstrated previously (Stone *et al*., 2012), we hypothesized that the synergistic effect of enzymes and antimicrobials towards *C. albicans* may be greater under aerobic conditions than anaerobic conditions. To test this hypothesis we studied binary interactions between strains of *C. albicans* and selected bacteria isolated from the same sewage polluted river environment, under both aerobic and anaerobic conditions. Additionally, we screened the bacteria for enzyme production and tested whether, in combination with prodigiosin, mannan degrading enzymes exert greater synergistic antifungal activity against unicellular growth of *C. albicans* than chitinases.

2.2 Materials and Methods

2.2.1 Sampling

Water samples were collected in sterile 1 L glass jars from the oxygen limited zone among plant debris close to the bank of the Plankenburg River (33°55'58.2"S 18°51'05.6"E, Western Cape, South Africa). This river flows through an industrial area and an informal settlement with inadequate sanitation infrastructure and an estimated population of 22 000 people (Paulse *et al*., 2009). The sampling site, situated ca. 1 km downstream of this informal settlement is exposed to pollution from both point and diffuse sources such as urban runoff, resulting in high levels of faecal pollution.

2.2.2 Bacterial isolation and identification

To isolate proteobacterial strains representing the Enterobacteriaceae, the water samples were serially diluted and spread onto MacConkey (Merck, Germany) agar and thereafter the plates were incubated for 24 h at 26ºC under aerobic and anaerobic conditions. The latter conditions were obtained with anaerobic chambers containing Anaerocult A (Merck, Germany) sets and were monitored with Anaerotest strips (Merck, Germany). Endospore forming bacteria, including *Clostridium* species, were isolated by the enrichment of endospores using Finley and Fields broth, incubated under aerobic or anaerobic conditions (Macagnan *et al*., 2006). After an incubation period of three days, cultures were heat treated for 10 min at 80ºC and then spread plates were prepared with Finley and Fields agar medium to obtain microbial colonies. Bacterial colonies were randomly selected from the isolation media and purified on agar before subsequent analyses. Once all the isolates were Gram stained (Prescott *et al*., 2008), the aerobic Gram positive, and oxidase negative Gram negative facultative anaerobic bacilli were tentatively identified using the API 50 CH test kit in combination with the API 50 CHB medium, as well as the API 20 E test kit respectively (bioMerieux, Marcy I'Etoile, France).

The identity of all bacterial isolates was confirmed using DNA sequence analyses of taxonomic informative gene sequences. For this purpose, genomic DNA was first extracted from each isolate by using the ZR Fungal/Bacterial DNA MiniPrep™ system (Zymo Research, California, USA) according to the manufacturer's instructions. The 16S (1500 bp)

region of the 30S small subunit ribosomal RNA was subsequently amplified by polymerase chain reaction (PCR) using universal ribosomal 16S primers (FWD 5′-AGTTTGATCCTGGCTCAG-3′ REV 5′-TACCTTGTTACGACTTCACCCCA-3), in an Applied Biosystems 2027 Thermal cycler (California, USA). The reaction consisted of 10 µl PCR master mix (2X) (Thermo Scientific, South Africa), 0.8 µl of each primer (Inqaba Biotechnical Industries, Pretoria, RSA), 1 µl DNA and 7.4 µl ddH_2O (double distilled water). The thermal cycling parameters were an initial denaturation at 95ºC for 1 min, followed by 30 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s, extension at 72ºC for 30 s, and final extension at 72ºC for 7 min. The PCR products were visualised under UV light (Geneflash Syngene Bioimaging Unit, Cambridge, UK) after electrophoresis in a 0.8 % (w/v) ethidium bromide containing agarose gel (Horizon 11.4 GIBCO DRL Horizontal Gel Electrophoresis Apparatus, Life Technologies, California, USA). Subsequently, PCR products were purified with Nucleospin® Extract II chromatography columns (Macherey-Nagel, Duren, Germany). Samples were sequenced using an ABI PRISM (model 3100) genetic sequencer (Applied Biosystems, California, USA). The sequences were compared to reported sequences using a BLAST search on the NCBI GenBank database (Altschul *et al*., 1990). Only sequences with ≥ 99% homology to known sequences were regarded as meaningful for identification to species level.

2.2.3 Yeast isolation and identification

One hundred milliliter aliquots of each water sample were filtered separately using cellulose nitrate filter disks (0.45 µm pore size) in sterile polycarbonate filter systems (Sartorius Sedim Biolab Products, Aubagne, France). The filter disks were transferred to Sabouraud glucose agar (SGA, pH 5.6) supplemented with 200 mg/l chloramphenicol (Sigma-Aldrich, Steinheim, Germany) and subsequently incubated for 48 h at 37ºC (Atlas, 1993). Yeast colonies were randomly selected and purified by repetitive subculturing on yeast malt (YM, pH 5.0) agar supplemented with 200 mg/l chloramphenicol. Pure yeast isolates were then tentatively identified by their colony color on Candiselect4 agar plates (Bio-Rad, Marnes-la-Coquette, France) that were incubated for 24 h at 30ºC. Purple colonies indicative of *C. albicans* were selected for identification using molecular methods. Putative *C. albicans* isolates were cultured in YM broth at 30ºC until log growth was reached. Yeast cells were harvested and genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep™

system (Zymo Research, California, USA) according to the manufacturer's instructions. The internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal rRNA gene operon (including the 5.8S gene) was subsequently amplified by PCR using the primer set ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). The D1/D2 (600–650 bp) region of the large subunit (26S) rRNA gene from each isolate was also amplified using the universal forward primer F63 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and reverse primer LR3 (5'-GGTCCGTGTTTCAAGACGG-3') (Fell *et al*., 2000). Amplification of the D1/D2 region, as well as the ITS regions, was performed in a 20 µl PCR mixture containing 10 µl PCR master mix (2X) (Thermo Scientific, South Africa), 0.8 µl of each primer (Inqaba Biotechnical Industries, Pretoria, RSA), 1 µl DNA and 7.4 µl ddH_2O. An initial denaturation step at 95ºC for 7 min was followed by 30 cycles, of denaturation at 95ºC for 30 s, annealing at 57ºC for 30 s followed by an elongation step at 72ºC for 1 min, carried out in an Applied Biosystems 2720 Thermal Cyler (California, USA). The final elongation step was conducted at 72ºC for 2 min and hereafter the PCR products were purified, sequenced and compared to reported sequences as described above.

2.2.4 Maintenance of microbial isolates

Using a numbering system starting with "CAB" all environmental and clinical microbial isolates were deposited in the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa (Tables 2.1 and 2.2). Clinical yeast strains (Table 2.1) were obtained from Tygerberg Hospital, Tygerberg, South Africa. Yeast isolates were stored in YM broth supplemented with 30% glycerol at -80°C and on YM agar slants. All yeast isolates were evaluated for typical characteristics of *C. albicans*, i.e. chlamydospore formation under anaerobic conditions (Nobile *et al*., 2003) and hyphal formation upon inoculation in egg white (Buckley and Uben, 1963). Bacterial isolates were stored in LB broth supplemented with 30% glycerol at -80°C and on agar slants containing LB.

2.2.5 Starter inoculums

Yeast and bacterial inocula were prepared by inoculating test tubes containing 5 ml LB broth and incubating them on a tissue roller drum (8 rev/min) in the dark. Cultures were allowed to grow for 16 h at 30ºC, thereafter the cells were harvested via centrifugation (12 000 × *g*, 5 min) in 2 ml microcentrifuge tubes. Each resulting pellet was washed thrice with physiological saline solution (PSS; 0.9% NaCl). Yeast and bacterial cells were enumerated using a haemocytometer (Improved Neubauer, Marienfeld Superior, Germany) and a Petroff-Hausser (Improved Neubauer, Marienfeld Superior, Germany) respectively, and then each suspension was diluted with PSS to produce the desired concentration as specified below. Unless otherwise specified, all yeast and bacterial inocula were prepared as described above.

2.2.6 Co-cultures

Four *C. albicans* isolates (Table 2.1) and 10 bacterial isolates (Table 2.2), were selected for co-culture experiments which were conducted in test tubes containing 5 ml LB broth (Tampakakis *et al*., 2009). Experiments were done in triplicate and each test tube was inoculated with log 5.67 yeast cells per ml and log 6 bacterial cells per ml. Test tubes were incubated under aerobic conditions on a tissue roller drum (8 rev/min) for 72 h at 26ºC. These co-culture assays were repeated under anaerobic conditions, which were maintained as described previously. Yeasts were subsequently enumerated on YM agar, containing 200 mg/l chloramphenicol (Sigma-Aldrich, Steinheim, Germany), using the spread plate technique. Plates were incubated for 48 h at 30ºC. The bacteria in each co-culture were enumerated using dilution plates prepared with LB medium that were incubated for 24 h at 26ºC. Pure cultures of *C. albicans* and bacteria were included as controls in each case. The pH of the cultures was determined at the end of the incubation period using a pH/temperature bench meter (Martini Instruments, Hungary). Prodigiosin concentrations were determined in both aerobic and anaerobic co-cultures containing *S. marcescens* CAB 1094 by first extracting the pigment with acetone according a method described in literature (Chang *et al*., 2000). Thereafter liquid chromatography mass spectrometry (LC–MS) was conducted on the extracts as described below.

2.2.7 Plate assays for extracellular bacterial enzymes

Bacterial isolates were screened, under both aerobic and anaerobic conditions, for extracellular hydrolytic enzyme activity while growing on agar media. In each case a sterile pipet tip was used to spot inoculate the middle of each agar plate, hereafter the cultures were incubated at 26ºC for ca. 48 h.

The ability to hydrolyze yeast cell walls was tested using plate assays prepared with solidified cell wall (CW) medium (Tanaka and Phaff, 1965) containing 0.2% (w/v) autolyzed and washed cells of *C. albicans* CAB 1084, 1.0% (w/v) K_2HPO_4, 0.001% (w/v) $MgSO_4 \cdot 7H_2O$ and 2.0% (w/v) bacteriological agar. The presence of hydrolytic enzymes, capable of yeast cell wall hydrolysis in this medium, was revealed by clear halos around bacterial colonies growing on an otherwise opaque background. Autolyzed and washed yeast cells were prepared as followed. Colonies of *C. albicans* CAB 1084 grown on YM agar plates, incubated at 30ºC for 48 h, were harvested by washing the cells from the plates using PSS. The cell suspension was subsequently washed thrice with PSS using centrifugation (15 000 × *g*, 15 min) where after the resulting pellet was suspended in 250 ml PSS. Toluene (500 ml) was added and the suspension was incubated at 55ºC for 72 h, where after it was centrifuged (15 000 × *g*, 15 min) and washed thrice with distilled water. The resulting pellet of ca. 150 g wet biomass was freeze dried, stored at 4ºC in a desiccator, and used as carbon and nitrogen source in the differential medium.

Chitinase production was examined using agar plates containing 0.5% (w/v) colloidal chitin (Ahmadi *et al*., 2008) prepared with chitin from shrimp shells (Sigma-Aldrich, Steinheim, Germany), 0.065% (w/v) Na_2HPO_4, 0.15% (w/v) KH_2PO_4, 0.025% (w/v) NaCl 0.05% (w/v) NH_4Cl, 0.012% (w/v) $MgSO_4$ and 0.0005% (w/v) $CaCl_2$. Chitinase production was revealed by clear halos around bacterial colonies growing on an otherwise opaque medium.

Mannanase activity was tested for using a differential medium containing 1.0% (w/v) mannan from *C. albicans* CAB 1084, 0.67% (w/v) filter sterilized Yeast Nitrogen Base, pH adjusted to 7 (Difco, YNB), and 2.0% (w/v) bacteriological agar. The mannan used in the medium was obtained via alkali extraction using a method adapted from literature (Castro *et al*., 1995; Gacto *et al*., 2000). Briefly, colonies of *C. albicans* CAB 1084 grown on YM agar plates, incubated at 30ºC for 48 h, were harvested by washing the cells from the plates using PPS. The cell suspension was subsequently washed thrice with PSS using centrifugation (15

000 × *g*, 15 min) resulting in ca. 130 g wet biomass. The mannan was then extracted twice with 6 % (w/v) NaOH for 90 min at 70ºC, and centrifuged (15 000 × *g*, 15 min). Subsequently, the mannan in the alkali-extracted supernatant was precipitated using Fehling's reagent (Algranati *et al*., 1966). Following centrifugation (15 000 × *g*, 15 min), the pellet was redissolved in cold 3 M HCl and precipitated using methanol. The final product was freeze dried, stored at 4 ºC in a desiccator, and used as carbon source in the differential medium. After incubation, the mannan containing plates were stained with 0.1% (w/v) Congo Red (B&M Scientific, South Africa) for 15 minutes and destained with 1 M NaCl for 15 minutes. Mannanase activity was revealed by clear halos around the bacterial colonies. Protease activity was tested for using skim milk agar (Atlas, 1993). The presence of protease was revealed by clear halos around bacterial colonies growing on an otherwise opaque medium.

2.2.8 Effect of extracellular bacterial enzymes on yeast cell wall

Enzyme cocktails from bacteria which tested positive for on chitin and/or mannan plate assays were further tested for their ability to release monomeric carbohydrates from a yeast cell wall suspension. Each enzyme cocktail was obtained by first inoculating the bacterial isolate into a test tube containing 5 ml CW broth and incubating it on a tissue roller drum (8 rev/min) for 48 h at 26ºC. Hereafter, 2 ml of the culture fluid of each bacterial isolate was harvested via centrifugation (12 000 × *g,* 5 min), filtered using a syringe filter (cellulose acetate, 0.2 µm pore size, GVS Filter Technologies, Bologna, Italy), and transferred to a test tube containing autoclaved 0.2% (w/v) autolyzed, washed cell walls of *C. albicans* CAB 1084. Test tubes were incubated for 48 h at 26ºC, which allowed potential extracellular hydrolytic enzymes to degrade the yeast cell walls. Following centrifugation (12 000 × *g,* 5 min) gas chromatography mass spectrometry (GC-MS) was used to test the supernatant, originating from the culture fluid of *A. hydrophila* CAB 1097, for mannose and *N*-acetylglucosamine, while the supernatant originating from the culture fluid of *K. pneumoniae* CAB 1101 was assayed for mannose (Roessner *et al*., 2001). These tests were conducted as explained below. Similarly, the sample from *S. marcescens* CAB 1094 was assayed for *N*-acetylglucosamine. In addition, extracellular enzyme cocktails from the culture fluids of all bacterial isolates were assayed for glucanase activity by testing for the presence of glucose originating from the yeast cell walls. A control consisting of supernatant from autoclaved 0.2% (w/v) autolyzed, washed cell walls of *C. albicans* CAB 1084 that was not treated with

an enzyme cocktail, was also included in the experimentation. Similar to the experimental tubes however, the control was incubated at 26ºC for 48 h before its reaction fluid was analysed with GC-MS for all three monomeric carbohydrates.

2.2.9 Presence of extracellular enzymes in co-cultures

A number of the above-mentioned yeast-bacterial co-cultures were screened, under both aerobic and anaerobic conditions, for extracellular hydrolytic enzyme activity. The yeast *C. albicans* CAB 1084 and three of the bacterial isolates, i.e. *A. hydrophila* CAB 1097, *K. pneumoniae* CAB 1101 and *S. marcescens* CAB 1094 were used in these experiments. In each case, 2 ml of the culture fluid was harvested via centrifugation (12 000 × *g,* 5 min) after the incubation period. The supernatant of each culture was subsequently filtered using a syringe filter (cellulose acetate, 0.2 µm pore size, GVS Filter Technologies, Bologna, Italy) and 500 µl of the filtrate was dripped onto CW agar plates. The plates were incubated at 26ºC for 16 to 72 h while being monitored for clear zones on the opaque agar where the filtrate was dripped onto. Supernatants from the yeast and bacterial monocultures were also assayed for enzyme activity on CW agar plates and all experiments were conducted in triplicate.

2.2.10 Fate of *C. albicans* in the presence of extracellular enzymes

Crude preparations of extracellular enzymes originating from *K. pneumoniae* CAB 1101 and *S. marcescens* CAB 1094 were prepared by growing each bacterial isolate at 26°C for 48 h in 25 ml LB medium contained in a 250 ml conical flask. In each case the culture fluid was harvested via centrifugation (15 000 × *g*, 10 min) and the supernatant was dialyzed at 4ºC for 24 h against 50 mM $NaPO_4$ (pH 7.0) using molecular porous dialysis tubing (Spectra/por 1 dialysis cellulose membrane, molecular weight cut off 6 000 - 8 000 Da, Spectrum medical industries Inc., Houston, TX). An aliquot (1.2 ml) of the resulting crude enzyme preparation, from either *K. pneumoniae* CAB 1101 or *S. marcescens* CAB 1094, was transferred to a 2 ml microcentrifuge tube. Washed cells of *C. albicans* CAB 1084 in exponential growth phase were subsequently suspended in each of the crude enzyme preparations to reach a final concentration of log 5.67 yeast cells per ml. The suspensions were then incubated at 26°C on

a tissue roller drum (8 rev/ min) for 2 h. Plate counts, to enumerate culturable yeasts, were subsequently conducted on YM agar incubated at 30°C.

2.2.11 Prodigiosin production

The broad spectrum antimicrobial compound, prodigiosin (2-methyl-3-pentyl-6-methoxyprodiginine), which is known to be produced by representatives of *S. marcescens* (Budavari *et al*., 1996; Williamson *et al*., 2006), was extracted from *S. marcescens* CAB 1094 during this study. Powdered peanut broth was prepared by adding ground and sieved unsalted peanuts to distilled water (20 g/l) and the suspension was autoclaved at 121ºC for 20 min (Giri *et al*., 2004). Subsequently, *S. marcescens* CAB 1094 was cultivated for 48 h at 26ºC in forty 250 ml conical flasks, each containing 25 ml of the powdered peanut broth. The cells were harvested via centrifugation (15 000 × *g*, 30 min) and the red pigment was extracted from the combined cell pellets with 300 ml acetone (Chang *et al*., 2000) After further evaporation, the crude preparation was purified by column chromatography using a silica gel column with hexane/ethyl acetate (9:1, by vol) as solvent. Ten ml fractions of the resulting eluent were collected in test tubes. Prodigiosin in these fractions was detected using thin layer chromatography (TLC) conducted with silica gel 70F254 plates and chloroform/methanol/water (80:15:1, by vol.) as solvent. All fractions forming a single red spot at a Rf value of 0.69 on the TLC plates (characteristic of prodigiosin) were combined, where after the solvent was evaporated to dryness. The presence and purity of prodigiosin in the dried product was determined using LC–MS as described below.

2.2.12 Fate of *C. albicans* in the presence of extracellular enzymes and prodigiosin

To test the synergistic effect of prodigiosin and bacterial extracellular enzymes on reducing yeast numbers, yeast suspensions in crude enzyme extracts were prepared and incubated at 26°C for 2 h on a tissue roller drum as described above. After the first hour of incubation, prodigiosin was added to each suspension resulting in a final concentration of 100 µg/ml. The suspensions were incubated for another hour before plate counts were used to enumerate viable yeasts able to grow on YM agar incubated at 30°C. All experiments described above

contained negative controls in which the crude enzyme extracts were first inactivated via boiling for 15 min, while all experiments were conducted at least in triplicate.

All crude enzyme preparations used to treat living cell suspensions of *C. albicans* CAB 1084 were also assayed for activity on CW agar plates to confirm the presence of hydrolytic enzymes, capable of yeast cell wall hydrolysis. In addition, plate assays, as described above, were used to determine the activity of chitinase, mannanase and protease in both the active and inactivated (via boiling for 15 min) crude enzyme preparations.

2.2.13 Chitin content of yeast cell wall

Two methods were used to determine whether the chitin content in the cell wall of *C. albicans* CAB 1084 differs between aerobic and anaerobic conditions. The first method is based on binding of a fluorescent dye to the chitinous layer in the yeast cell wall; subsequently the staining intensity is estimated using fluorescence microscopy. For this purpose, *C. albicans* CAB 1084 was first grown in LB broth, incubated at 26ºC for 72 h, under both aerobic and anaerobic conditions. Cells were harvested by centrifugation (12 000 × *g*, 5 min) washed three times with PSS and stained with calcofluor white (Sigma-Aldrich, Steinheim, Germany). Fluorescence microscopy conducted with a Carl Zeiss Confocal LSM 780 Elyra S1 and SR-SIM superresolution platform was used to capture cellular images. Relative fluorescence intensity of the yeast cell walls were determined using ZEN microscope software (version 2011) (Germany). The relative intensity of 60 random spots on the cell wall of 60 different aerobic and anaerobic cells was used to calculate the average relative intensity. Spots were never chosen near yeast bud scars due to the higher chitin content in bud scars (Cabib and Bowers, 1971).

The second method used to determine whether the chitin content in the cell wall of *C. albicans* CAB 1084 differs between aerobic and anaerobic conditions, is based on estimating the *N*-acetylglucosamine content of the cell wall using chemo-analytical techniques (Bulawa *et al*., 1986). Briefly, *C. albicans* CAB 1084 was grown, under both aerobic and anaerobic conditions, in test tubes containing LB broth for 72 h at 26°C. Washed cells (100 mg) were then suspended in 1 ml of 6% KOH and heated for 90 min at 60ºC. After addition of 0.1 ml of glacial acetic acid, the insoluble material was collected by centrifugation (4 000 × *g*, 5 min), washed twice with water, and resuspended in 0.5 ml buffer containing 50 mM $NaPO_4$

(pH 6.3). Subsequently 40 µl of partially purified chitinase, consisting of 5 mg/ml *Streptomyces griseus* chitinase (Sigma-Aldrich, product code C6137, Steinheim, Germany) dissolved in 50 mM $NaPO_4$ (pH 6.3) was added to the cell suspension and incubated for 1 hour at 37ºC. Following centrifugation (12 000 × *g*, 15 min), 400 µl of the supernatant was treated with 40 µl of cytohelicase, consisting of 50 mg/ml *Helix pomatia* cytohelicase (Sigma-Aldrich, product code C8274, Steinheim, Germany) dissolved in 50 mM sodium citrate buffer (pH 4.6) for 1 hour at 37ºC. A 100 µl portion of each sample was removed and prepared for GC-MS analysis and assayed for *N*-acetylglucosamine as described below.

2.2.14 Mannan content of yeast cell wall

Two different methods were used to determine mannan levels in the cell wall of *C. albicans* CAB 1084 while growing under aerobic and anaerobic conditions. For the first method, *C. albicans* CAB 1084 was grown in LB broth, incubated at 26ºC for 72 h, under both aerobic and anaerobic conditions. Cells were harvested by centrifugation (12 000 × *g*, 5 min) washed three times with PSS and stained with concanavalin A, fluorescein conjugate (Life Technologies, product code C827). Fluorescence microscopy was performed in a similar manner as for the calcofluor white stained cells. Using the same software as described above the relative fluorescence intensity of the entire mannan layer from each of 60 random aerobically grown cells, as well as that from 60 random anaerobically grown cells, were used to calculate the average relative intensity for aerobically and anaerobically grown cells respectively.

The second method used to determine the mannan levels in the yeast cell wall is based on estimating the mannose content by employing GC-MS analyses. *Candida albicans* CAB 1084 was grown, under both aerobic and anaerobic conditions, in test tubes containing LB broth for 72 h at 26°C. After incubation, the cells were harvested via centrifugation, washed thrice, and then total hydrolysis was done and cells were prepared for GC-MS analysis. Ribitol (100 µl) was added in the samples as internal standard before sample hydrolysis. Two millilitres of samples were hydrolyzed with 2 ml of 2 M trifluroacetic acid (TFA) at 121°C for 2 hours. Subsequently, the samples were cooled and centrifuged (8 000 ×*g*, 10 min), and thereafter the TFA resistant residue was washed twice with 2 M TFA. The resulting supernatants were pooled, and TFA evaporated in the Genevac® EZ2 personal evaporator. To remove the

residual TFA, samples were dissolved in 1 ml methanol and evaporated as described before. This process was repeated three times and samples were examined using GC-MS analysis as described below.

2.2.15 Determining prodigiosin concentration using LC–MS

Analyses were performed using a Waters (Milford, MA, USA) Synapt G2 quadrupole time of flight mass spectrometer coupled to a Waters Acquity ultra performance liquid chromatograph (UPLC) fitted with an Acquity photo diode array detector (Long *et al*., 2012). Separation was achieved with a Waters HSS column (C18, 2.1 × 150 mm, 1.7 µm particle size) using mobile phases consisting of A: water and B: acetonitrile at a flow rate of 0.3 ml per min. The gradient that was employed started for the first 0.5 min at 100% solvent A followed by a linear gradient to 100% B over the next 13 min. The column was subsequently re-equilibrated for 1.9 min with 95 % of A and 5 % of B. Mass spectrometry (MS) was employed by applying electrospray ionization in the positive mode at a capillary voltage of 2.5 kV, cone voltage of 15 V, desolvation temperature of 275 °C and desolvation gas setting of 650 L/h (Long *et al*., 2012). The rest of the MS settings were optimized to achieve the best sensitivity. The instrument was calibrated with sodium formate while leucine enkephalin was used as lock mass for accurate mass determinations. Prodigiosin was detected at 4.27 min with a *m/z* of 324.2067 which corresponds to its [M+H] ion.

2.2.16 Determining monomeric carbohydrate concentrations using GC-MS

Aqueous samples containing monomeric carbohydrates were allowed to evaporate for 14 h in a Genevac® EZ2 personal evaporator. The remaining residue was dissolved and derivatized for 2 h at 30°C in 40 µl 20 mg/ml methoxyamine hydrochloride (Sigma-Aldrich, Steinheim, Germany), which was followed by a 30 min treatment with 70 µl *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, Steinheim, Germany) at 37°C. Ribitol was included as an internal standard. Sample volumes of 1 µl were subsequently injected into a gas chromatograph (Trace 1300; Thermo scientific) coupled to a Thermo TSQ 8000 GC-MS/MS equipped with a TriLusRSH Autosampler and a non-polar (95% dimethylpolysiloxane) capillary column (TG-SQC, No. 26070-1300, Thermo Scientific; 15

m, 0.25 mm ID, 0.25 µm film thickness). The oven temperature program was as follows: the initial oven temperature was maintained at 100°C for 4 min, then ramped at 6°C/min to 180°C and held for 2 min, where after a final ramp at 15°C/min to 300°C and then a last held for 3 min. Helium was used as carrier gas at a flow rate of 1 ml/min and the injector temperature was maintained at 280°C operated in splitless mode. The mass spectral data was recorded on the TSQ operated in a single reaction mode (SRM) with the ion source temperature set at 240°C. The transfer line temperature was maintained at 280°C. Carbohydrate identification was based on comparisons with standard mass spectra in the MS spectral library. The presence of mannose and glucose was confirmed by comparing the mass spectra of their oxime-TMS derivatives, characterized by the ions m/z 73, 147, 205 and 319, whereas the presence of *N*-Acetyl glucosamine was confirmed by the presence of ions *m/z* 217, 319 and 333. For quantification purposes, the ion at *m/z* 319 was used for both mannose and glucose, while *m/z* 333 was selected to quantify *N*-Acetyl glucosamine. The calibration curve was plotted by the ratio obtained from dividing the areas of the respective sugars with the area of ribitol. The concentrations of the sugars in the samples were read against the calibration curves.

2.3 Results

2.3.1 Yeast and bacterial isolates

We randomly selected two yeast isolates representing *C. albicans* (Table 2.1), and 10 bacterial isolates, representing the Firmicutes and Proteobacteria (Table 2.2), from all the isolates obtained from water among plant debris in the Plankenburg River. The bacterial isolates representing the Proteobacteria were all facultative anaerobes belonging to the families Aeromonadaceae and Enterobacteriaceae, while those representing the Firmicutes were either aerobic bacilli or anaerobic clostridia. (Gram staining results and phylogenetic tree analysis depicted in Fig. S.1-3; Appendix A)

TABLE 2.1 Clinical and environmental yeasts isolates used in this study.

***C. albicans* isolate**	**Origin, clinical/environmental isolate**	**GenBank accession no.**
CAB 1084	Plankenburg River, environmental	KJ534502
CAB 1085	Plankenburg River, environmental	KJ534503
8908	Tygerberg Hospital, South Africa, clinical	KJ534504
8912	Tygerberg Hospital, South Africa, clinical	KJ534505

2.3.2 Fate of *C. albicans* in the presence of different bacteria

The survival of the four *C. albicans* isolates, after 72 h at 26ºC, in co-cultures prepared separately with each of the eight aerobic or facultative anaerobic bacterial isolates, is depicted in Fig. 2.1. Under aerobic conditions the bacteria exerted a notable negative effect on *C. albicans* and no culturable yeast cells were detected in co-cultures with *A. hydrophila* CAB 1097. Also, the presence of the prodigiosin producing bacterium *S. marcescens* CAB 1094 resulted in growth inhibition of all four yeast strains. Subsequent microscopic analyses revealed the presence of this red pigment inside the yeast cells (Fig. 2.2a) However, when we conducted these co-culture experiments under anaerobic conditions, we detected no visible pigment in the yeasts within the *S. marcescens* co-cultures (Fig. 2.2b). LC-MS analysis revealed that after 72h the prodigiosin concentration in the aerobic co-cultures containing *S. marcescens* CAB 1094 was 11.33 ± 0.3 µg/ml. Prodigiosin was not detectable in the anaerobic co-cultures containing *S. marcescens* CAB 1094. Under anaerobic conditions yeast numbers generally appeared to be unaffected by the presence of the facultative anaerobe bacterial isolates (Fig. 2.3). Likewise, none of the *Clostridium* species seem to have an effect on the proliferation of *C. albicans* CAB 1084 (Fig. 2.3). The pH of all aerobic cultures (co-cultures and monocultures) increased from 7.4 to ca. 8.5 after 72 h of incubation, (Fig. S.4; Appendix A). In contrast, the pH of all the anaerobic cultures decreased to ca. pH 6.5.

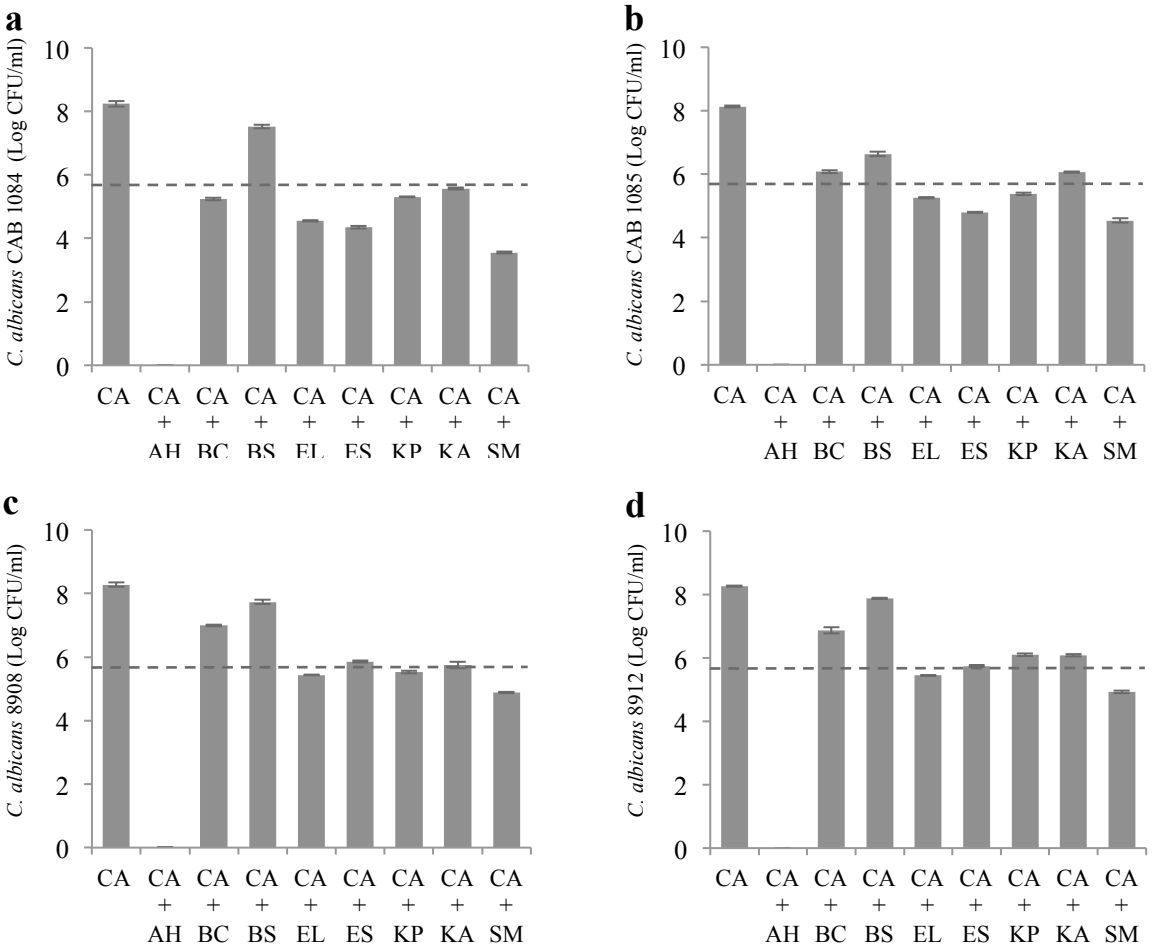

Fig. 2.1 Concentrations of, **a** *Candida albicans* CAB 1084, **b** *C. albicans* CAB 1085, **c** *C. albicans* 8908, and **d** *C. albicans* 8912, when co-cultured separately with each of the eight bacterial isolates for 72 h at 26ºC under aerobic conditions. AH, *Aeromonas hydrophila* CAB 1097; BC and BS, *Bacillus* isolates (CAB 1112 and CAB 1111); EL and ES, *Enterobacter* isolates (CAB 1099 and CAB 1098); KP, *Klebsiella pneumoniae* CAB 1101; KA, *Kluyvera ascorbata* CAB1103; SM, *Serratia marcescens* CAB1094. In each case the first bar represents the control, *C. albicans* monoculture. Bars represent the mean of three repetitions and the whiskers indicate the standard error. The dashed line represents the initial concentration of the yeasts. The co-cultured yeast numbers were statistically less than that of the control in all cases (Fisher LSD test, $p \leq 0.05$; Statistica Version 12, StatSoft).

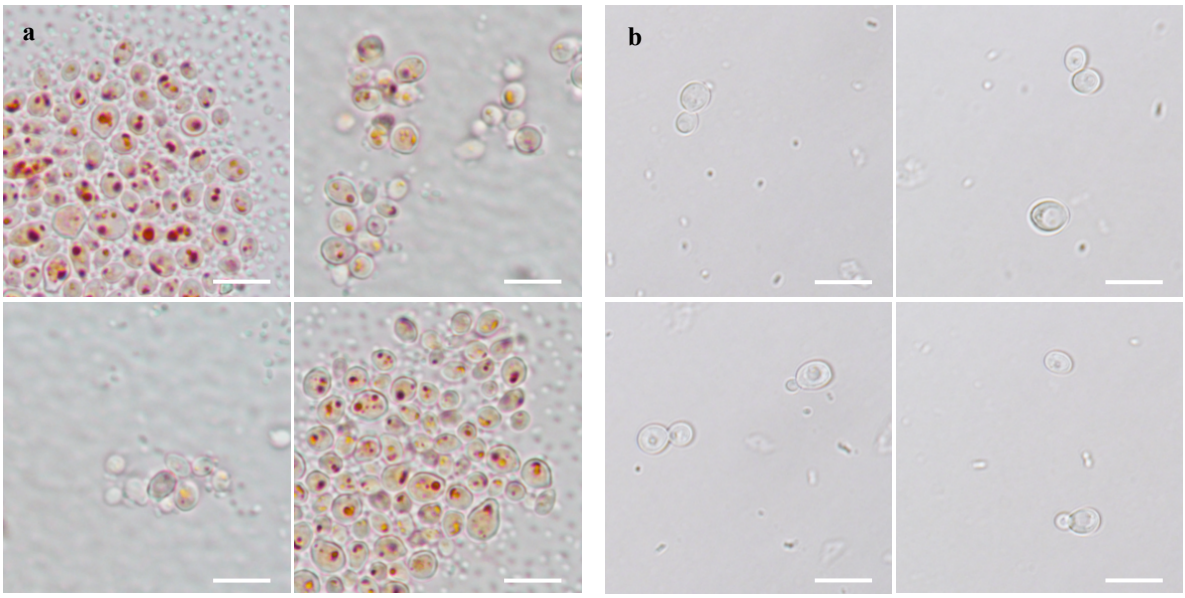

Fig. 2.2 Light microscopy images of *C. albicans* CAB 1084 cells grown in co-culture with *S. marcescens* CAB 1094 for 72 h at 26ºC in LB broth. In aerobically grown co-cultures the red pigment, prodigiosin is visible inside the *C. albicans* cells (Panel **a**). However, in anaerobic co-cultures, no pigment is visible inside the yeast cells (Panel **b**). Light microscopy images were conducted using a Nikon eclipse E400 microscope equipped with a Nikon DS-Fi2 camera and a Nikon Digital Sight DS-U3 camera controller (Nikon, Japan). The scale bar represents 10 µm.

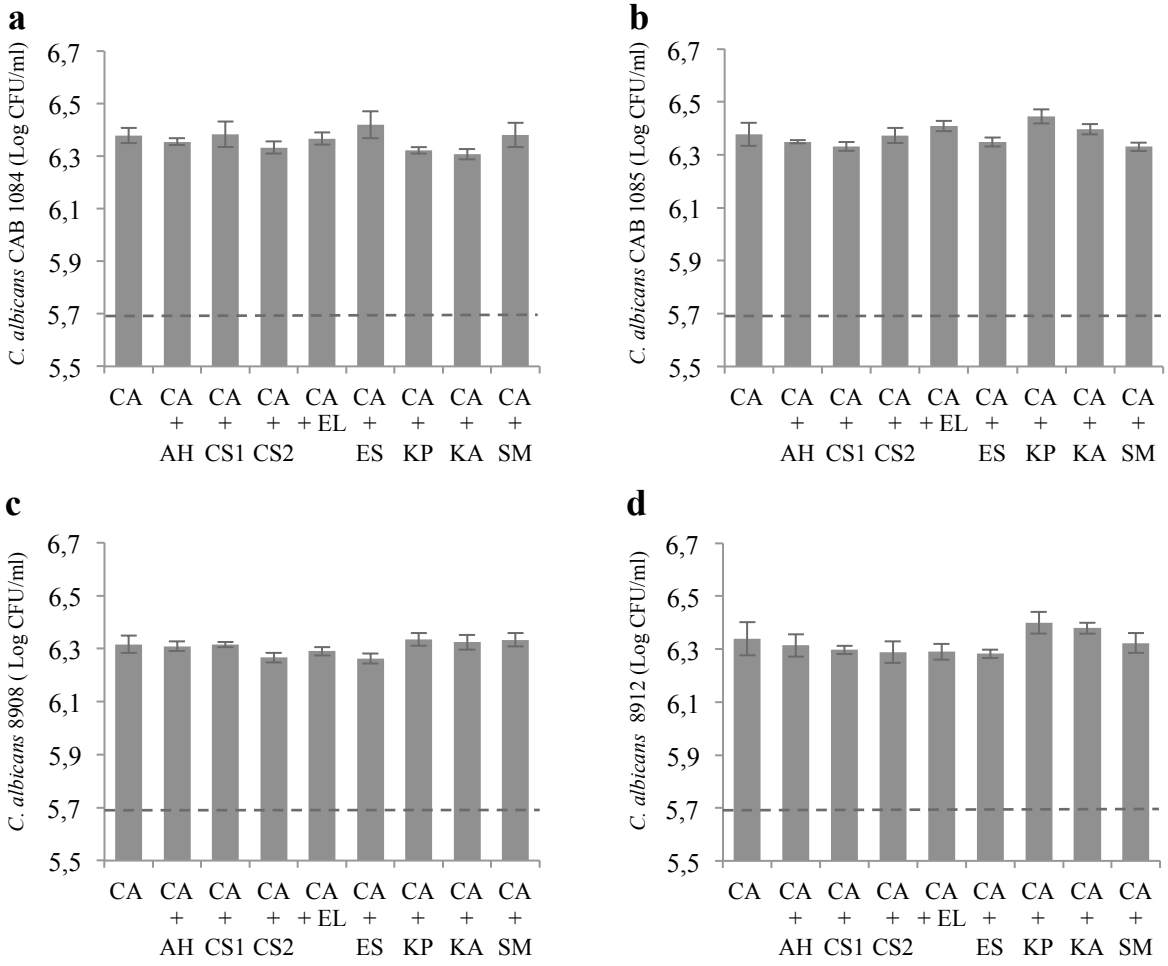

Fig. 2.3 Concentration of, **a** *Candida albicans* CAB 1084, **b** *C. albicans* CAB 1085, **c** *C. albicans* 8908, and **d** *C. albicans* 8912, when co-cultured with eight bacterial isolates for 72 h at 26ºC under anaerobic conditions. AH, *Aeromonas hydrophila* CAB 1097; CS1 and CS2, *Clostridium* isolates (CAB 1115 and CAB 1116); EL and ES, *Enterobacter* isolates (CAB 1099 and CAB 1098); KP, *Klebsiella pneumoniae* CAB 1101; KA, *Kluyvera ascorbata* CAB1103; SM, *Serratia marcescens* CAB1094. In each case the first bar represents the control, *C. albicans* monoculture. Bars represent the mean of three repetitions and the whiskers indicate standard error. The dashed line represents the initial concentration of the yeast in the co-culture. In all cases none of the co-culture yeast concentrations was significantly less than the control (Fisher LSD test, $p > 0.05$; Statistica Version 12, StatSoft).

2.3.3 Bacterial numbers in the presence of *C. albicans* CAB 1084

Bacterial cell concentrations of the eight aerobic and facultative anaerobic bacteria, after 72 h at 26ºC, in aerobic co-cultures prepared with *C. albicans* CAB 1084 are depicted in Fig. 2.4a. Aerobic bacterial growth seemed to proceed unhindered in the presence of the yeast, and was in fact enhanced in the *C. albicans* CAB 1084/*K. pneumoniae* CAB 1101 co-culture. Similarly, anaerobic growth of the facultative anaerobe bacterial and the *Clostridium* isolates seemed to be generally unaffected by the presence of *C. albicans* CAB 1084 (Fig. 2.4b).

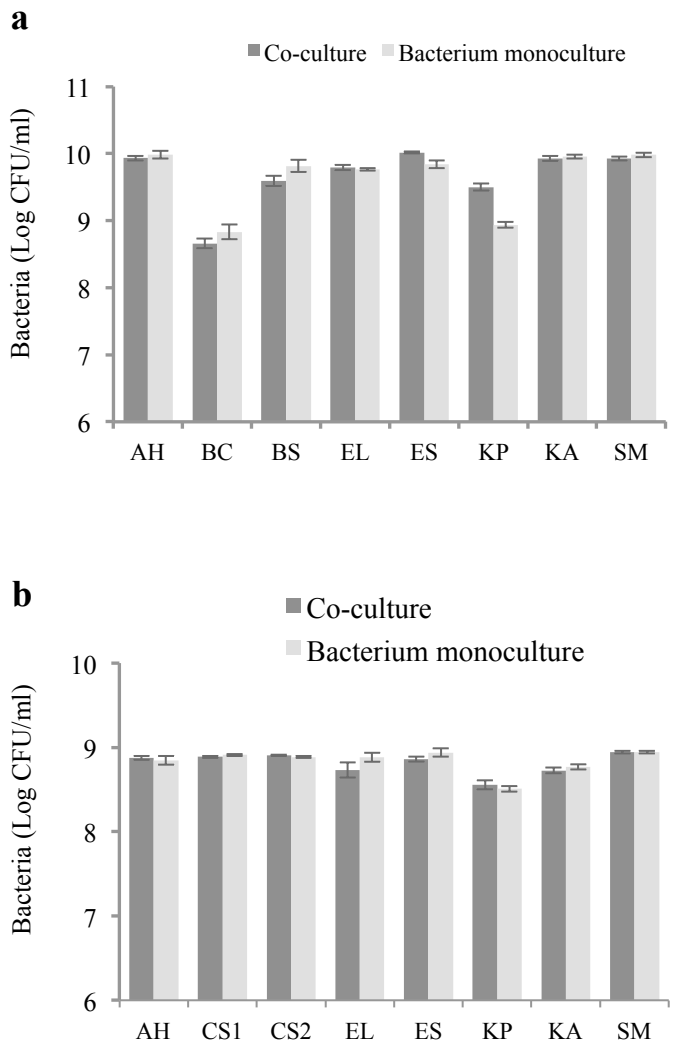

Fig. 2.4 Concentrations of, **a** the eight aerobic and facultative anaerobic bacteria, and **b** the facultative anaerobe bacterial isolates, as well as the clostridia when co-cultured with *C. albicans* CAB 1084 for 72 h at 26ºC under aerobic and anaerobic conditions respectively.

AH, *Aeromonas hydrophila* CAB 1097; BC and BS, *Bacillus* isolates (CAB 1112 and CAB 1111); CS1 and CS2, *Clostridium* isolates (CAB 1115 and CAB 1116); EL and ES, *Enterobacter* isolates (CAB 1099 and CAB 1098); KP, *Klebsiella pneumoniae* CAB 1101; KA, *Kluyvera ascorbata* CAB1103; SM, *Serratia marcescens* CAB1094. Bars represent the mean of three repetitions and the whiskers indicate standard error. None of the co-culture bacteria concentrations was significantly less than the monocultures (Fisher LSD test, $p > 0.05$; Statistica Version 12, StatSoft).

2.3.4 Extracellular bacterial enzyme production

With the exception of *K. ascorbata* CAB 1103, all eight aerobic and facultative anaerobic bacterial isolates that were screened, formed clear halos around their colonies when grown on CW agar medium under aerobic conditions (Table 2.2). Only one isolate, *A. hydrophila* CAB 1097 formed a clear halo on CW medium under anaerobic conditions. Under aerobic conditions we observed chitinase activity on chitin containing plates that were inoculated with *A. hydrophila* CAB 1097 and *S. marcescens* CAB1094, while the colonies of all bacteria, except the *Enterobacter* strains, showed proteinase activity on the skim milk containing plates (Table 2.2). Both *A. hydrophila* CAB 1097 and *K. pneumoniae* CAB 1101 showed mannanase activity under aerobic conditions. Except for chitinase produced by *A. hydrophila* CAB 1097, none of these enzymes were detectable under anaerobic conditions. Similarly, neither of the two *Clostridium* isolates showed clear halos around their colonies on the CW agar plates. Subsequent GC-MS analyses of cell wall suspensions that we treated with enzyme cocktails originating from *A. hydrophila* CAB 1097 and *K. pneumoniae* CAB 1101, showed the presence of mannose (the hydrolysis product of mannan). Also, enzyme cocktails from *A. hydrophila* CAB 1097 and *S. marcescens* CAB 1094 produced a cell wall suspension containing *N*-acetylglucosamine, the monomer of chitin. Neither mannose, nor *N*-acetylglucosamine was detectable in the control. Glucose was not detectable in any of the samples, including the control, indicating that the cell wall glucans were not hydrolyzed.

Table 2.2 The morphological and physiological characteristics of the bacterial isolates which represent phylogenetic unrelated species.

Bacterial isolates	GenBank accession no.	Gram reaction	Bacterial Phylum	Oxygen requirements	Enzyme activity[a]							
					Cell Wall Enzymes		Chitinase		Mannanase		Protease	
					Aer	Ana	Aer	Ana	Aer	Ana	Aer	Ana
Aeromonas hydrophila CAB 1097	KJ194588	-	P	Fa	+	+	+	+	+	-	+	-
Bacillus cereus CAB 1112	KJ194589	+	F	A	+	/	-	/	-	/	+	/
Bacillus subtilis CAB 1111	KJ194590	+	F	A	+	/	-	-	-	/	+	-
Enterobacter ludwigii CAB 1099	KJ194592	-	P	Fa	+	-	-	-	-	-	-	-
Enterobacter sp CAB 1098	KJ194591	-	P	Fa	+	-	-	-	-	-	-	-
Klebsiella pneumoniae CAB 1101	KJ194593	-	P	Fa	+	-	-	-	+	-	+	-
Kluyvera ascorbata CAB 1103	KJ194594	-	P	Fa	-	-	-	-	-	-	+	-
Serratia marcescens CAB 1094	KJ194595	-	P	Fa	+	-	+	-	-	-	+	-
Clostridium sp CAB 1115	KJ194596	+	F	An	/	-	/	-	/	-	/	+
Clostridium sp CAB 1116	KJ194597	+	F	An	/	-	/	-	/	-	/	+

[a] Enzyme activity was tested at 26ºC under both aerobic and anaerobic conditions using different plate assays. Positive results were indicated by clear halos around bacterial colonies growing on an otherwise opaque medium.

F, Firmicutes; P, Proteobacteria; A, Aerobe; An, Anaerobe; Fa, Facultative anaerobe; Aer, Aerobic conditions; Ana, Anaerobic conditions; +, Positive for enzyme production; -, Negative for enzyme production; /, Does not grow under the specified conditions.

2.3.5 Presence of extracellular enzymes in co-cultures

The CW plate assay revealed extracellular enzyme activity in the cell free culture fluids from aerobic monocultures of *A. hydrophila* CAB 1097, *K. pneumoniae* CAB 1101 and *S. marcescens* CAB 1094, as well as in the culture fluids originating from aerobic co-cultures of *C. albicans* CAB 1084 with these bacteria (Results not shown). Except for the culture fluids originating from anaerobic cultures prepared with *A. hydrophila* CAB 1097, none of the culture fluids from the anaerobic cultures showed enzyme activity on CW plates (Results not shown). No enzyme activity was detectable in monocultures of *C. albicans* CAB 1084.

2.3.6 Fate of *C. albicans* in the presence of extracellular enzymes and prodigiosin

Figure 2.5 depicts the survival of *C. albicans* CAB 1084 in each of the crude extracellular enzyme preparations, originating from *K. pneumoniae* CAB 1101 and *S. marcescens* CAB 1094. The crude enzyme preparations did not exert a significant negative effect on the cell numbers of *C. albicans* CAB 1084. However, 100 µg/ml prodigiosin, extracted from *S. marcescens* CAB 1094 (ca. 37.5 mg/g biomass after cultivation in powdered peanut broth), showed a negative effect on yeast numbers in the presence of inactivated enzyme extracts. This negative effect of prodigiosin was more pronounced in the presence of the active enzyme extract from *K. pneumoniae* CAB 1101.

Hydrolytic activity assays of the crude enzyme preparations, used to treat living cell suspensions of *C. albicans* CAB 1084, on CW agar plates, indicated that all active crude preparations formed clear zones on the plates, while the inactivated crude preparations showed no reaction. In addition, the active crude enzyme preparations from *K. pneumoniae* CAB 1101 and *S. marcescens* CAB 1094 showed protease activity on skim milk plates, while the enzyme preparation from *S. marcescens* CAB 1094 showed chitinase activity on a chitin containing plate. Furthermore, the active crude enzyme preparation of *K. pneumoniae* CAB 1101 showed mannanase activity on a mannan containing plate.

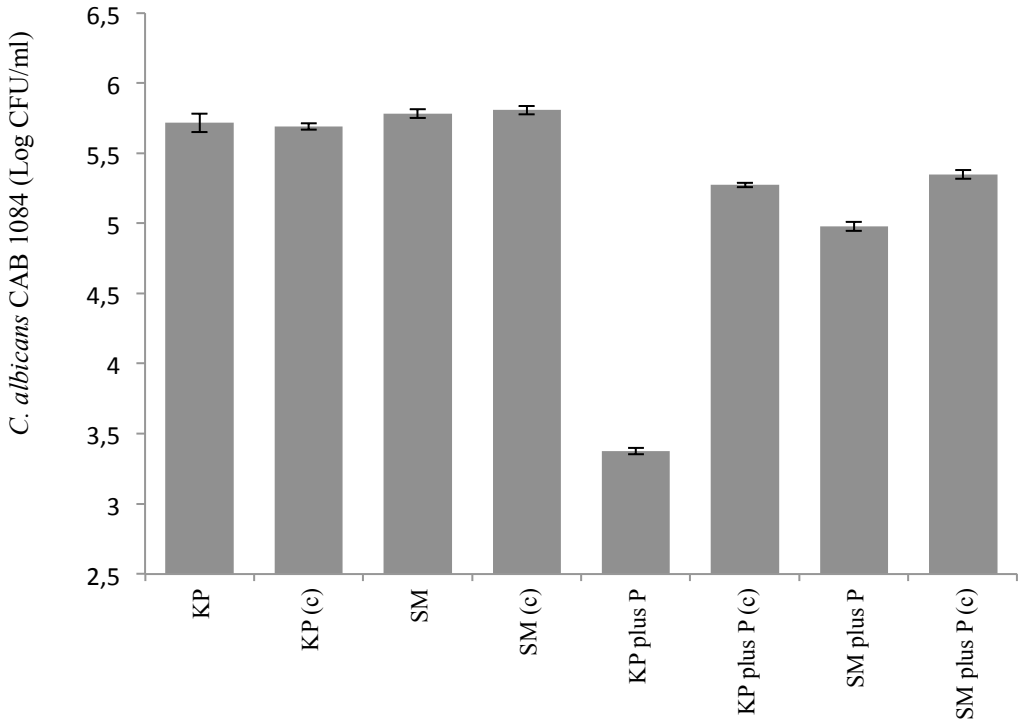

Fig. 2.5 Concentrations of culturable *Candida albicans* CAB 1084 cells suspended for 2 h in dialysed crude extracellular enzyme extracts of *Klebsiella pneumoniae* CAB 1101 (KP) and *Serratia marcescens* CAB 1094 (SM), either in the absence or presence of 100 µg/ml prodigiosin (P). Inactivated enzyme extract represents the control (c) in each case. No significant difference was observed between the results obtained with dialysed enzyme extracts compared to the inactivated dialysed controls. Compared to these controls, significantly less culturable yeast cells were recovered from both the active and inactivated crude extracellular enzyme extracts when 100 µg/ml prodigiosin was present in the yeast suspensions. Also, prodigiosin in combination with active enzyme extracts had a greater negative effect on culturable yeast cell numbers than prodigiosin and inactivated enzyme extracts. Bars represent the mean of three repetitions and the whiskers indicate standard error. (Fisher LSD test, $p \leq 0.05$; Statistica Version 12, StatSoft).

2.3.7 Chitin content of yeast cell wall

Fluorescence microscopy revealed that the relative intensity of aerobically grown, calcofluor stained *C. albicans* CAB 1084 cells was 38.72 ± 0.85 (SE), significantly more than the relative intensity of 22.56 ± 0.5 (SE) recorded for the anaerobically cultured yeast cells (Fig.

2.6). Subsequent chemical analyses of the yeast cell walls supported these findings, where *C. albicans* CAB 1084 cells contained 30.59 ± 2.21 (SE) µg *N*-acetylglucosamine /100 g wet cells, and 11.98 ±1.42 µg (SE) *N*-acetylglucosamine /100 g wet cells when cultured under aerobic and anaerobic conditions respectively.

2.3.8 Mannan content of yeast cell wall

Fluorescence microscopy revealed that the relative intensity of aerobically grown, concanavalin A stained *C. albicans* CAB 1084 cells was 51.38 ± 1.69 (SE), which was not significantly different than the relative intensity of 57.38 ± 1.05 (SE) recorded for the anaerobically cultured yeast cells (Fig. 2.6). Subsequent chemical analyses of the yeast cell walls supported these findings, where *C. albicans* CAB 1084 cells contained 214.77 ± 4.02 (SE) µg mannose/100 g wet cells, and 209.29 ± 4.39 µg (SE) mannose /100 g wet cells when cultured under aerobic and anaerobic conditions respectively.

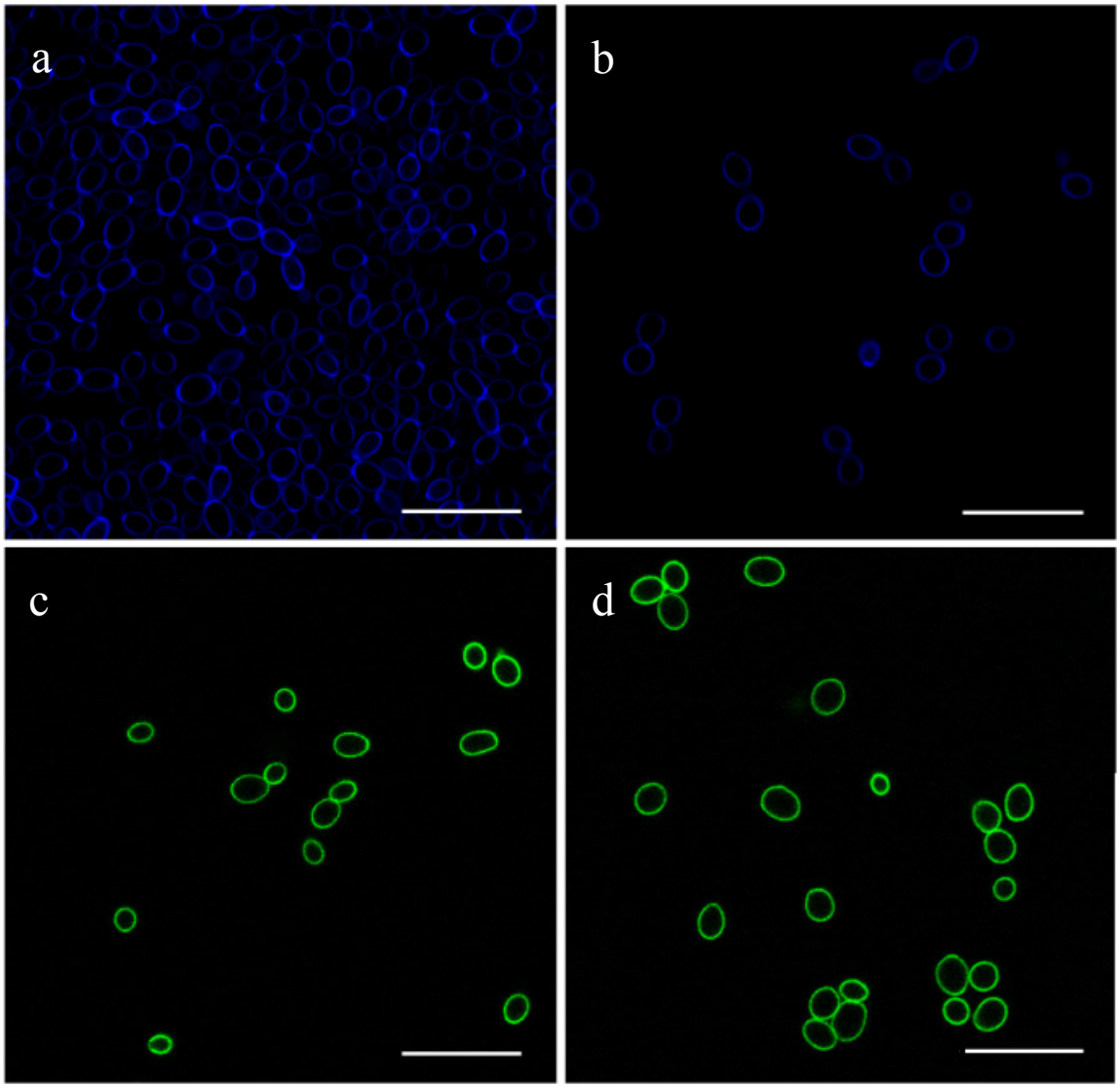

Fig. 2.6 Confocal images of calcofluor white and concanavalin A, fluorescein conjugate stained *C. albicans* CAB 1084 cells. **a** calcofluor white stained cells grown aerobically for 72 h at 26ºC in LB broth had a relative intensity of 38.72 ± 0.85 (SE) and, **b** cell walls of anaerobically cultured yeast cells had a relative intensity of 22.56 ± 0.5 (SE). **c** concanavalin A, fluorescein conjugate stained cells grown aerobically for 72 h at 26ºC in LB broth had a relative intensity of 51.38 ± 1.69 (SE), and **d** cell walls of anaerobically cultured yeast cells had a relative intensity of 57.38 ± 1.05 (SE). The scale bar represents 20 µm.

2.4 Discussion

Our results indicate that bacteria may exert a negative effect on the survival of *C. albicans* under aerobic conditions, while this antagonistic effect is ameliorated under anaerobic conditions. In contrast, it seems that under both aerobic and anaerobic conditions, *C. albicans* had no negative effect on bacterial growth, as observed in co-cultures prepared with *C. albicans* CAB 1084. Interestingly, it appears that growth of *K. pneumoniae* CAB 1101 was enhanced in the presence of *C. albicans* CAB 1084. A factor contributing to this phenomenon may be the ability of *K. pneumoniae* CAB 1101 to produce enzymes capable of hydrolyzing α-mannan, since it is known that some bacteria can utilize α-mannan as carbon source (Cuskin *et al*., 2015).

Although the anaerobic and aerobic cultures differed with regard to pH, the observed antagonistic effect on culturable yeast cells seemed not to be the result of changes in the pH of the culture media, since similar pH changes as a result of microbial metabolic activity (Han and Lee, 2006; Vylkova *et al*., 2011) occurred in both the co- and monocultures. However, microscopic analyses after 72 h of incubation indicated the presence of the antifungal agent, prodigiosin, within yeast cells of the aerobic *C. albicans*-*S. marcescens* co-cultures, while some other aerobic co-cultures comprised mainly of bacterial cells and yeast cell debris which is indicative of degradative enzyme activity.

Subsequent enzyme assays confirmed that under aerobic conditions, 88% of the aerobic and facultative anaerobic bacterial isolates showed extracellular hydrolytic enzyme activity towards yeast cell wall polymers. However, under anaerobic conditions only one bacterial isolate, *A. hydrophila* CAB 1097, was capable of producing cell wall hydrolyzing enzymes (Table 2.2). This apparent absence of bacterial hydrolytic enzyme production under anaerobic conditions may have been the result of energy limitations in the absence of oxygen (Weber *et al*., 2006). Studies previously demonstrated that the expression of more than one-third of the genes expressed during aerobic growth is altered when *Escherichia coli* cells are shifted to anaerobic conditions (Salmon *et al*., 2003). In addition, we confirmed that the pigmented antifungal compound, prodigiosin, was not produced under anaerobic conditions thereby supporting the findings of others (Khanafari *et al*., 2006; Williams *et al*., 1971).

Despite evidence in literature that bacterial extracellular hydrolytic enzymes, such as chitinase, may exert antifungal activity against *C. albicans* (Bao-qin *et al*., 2004; Han *et al*.,

2009), no such antifungal activity was detected during the current study, when *C. albicans* CAB 1084 was anaerobically grown in the presence of the chitinase producing bacterium, *A. hydrophila* CAB 1097. In addition, we found no detrimental effect on the numbers of *C. albicans* CAB 1084 when cells of this yeast were suspended in crude preparations of extracellular enzymes originating from aerobic cultures of *K. pneumoniae* CAB 1101 or *S. marcescens* CAB 1094 (Fig. 2.5). However, in the presence of these crude preparations and prodigiosin from *S. marcescens* CAB 1094, we observed a significant decrease in the numbers of culturable yeast cells. These results support the findings of others who discovered that a number of fungi, including *Didymella applanata* (Shternshis *et al*., 2006), *Fusarium oxysporum* (Someya *et al*., 2007), *Trichoderma reesei* and *Phycomyces blakesleeanus* (Roberts and Selitrennikoff, 1988), are relatively resistant *in vitro* to the effect of *S. marcescens* chitinases. However, studies indicate that the co-inoculation of *S. marcescens* and *Pseudomonas fluorescens* enhances the antifungal activity of *P. fluorescens* towards *F. oxysporum* (Someya et al., 2007), and it was contented that this enhanced antifungal effect was a result of *S. marcescens* chitinase activity. Similarly, researchers found that culture filtrates from *S. marcescens* show synergistic antifungal activity with a number of chemical fungicides against sclerotial viability of *Rhizoctonia solani* (Someya *et al*., 2005). Also, others found that the antifungal properties of different fungitoxic compounds against *Botrytis cinerea* are synergistically enhanced by the addition of cell wall degrading enzymes, such as chitinases or glucanases, to the reaction mixture (Lorito *et al*., 1994).

Interestingly, our findings indicated that the antagonistic effect of prodigiosin was more pronounced in the presence of crude enzyme preparations of *K. pneumonia* CAB 1101 which showed only mannanase activity, compared to crude preparations containing chitinase without mannanase activity from *S. marcecens* CAB 1094 (Table 2.1, Fig. 2.5). This phenomenon may be ascribed to the fact that unlike chitin, which is a structural polysaccharide of the inner cell wall, the mannans of the outer cell wall are less structured and have low permeability and porosity (Gow and Hube, 2012), thereby protecting the cell against antifungal agents. Mannanases produced by bacteria may thus influence the permeability of the cell wall of *C. albicans*, thereby increasing the susceptibility to antifungals such as prodigiosin.

Similar to previous findings on the cell wall composition of *Saccharomyces cerevisiae* (Aguilar-Uscanga and Francois, 2003), we found that the chitin content of *C. albicans* cell

walls decreased, while the mannan content remained the same under anaerobic conditions, when compared to aerobic conditions. This decreased chitin content under anaerobic conditions does not seem to increase the susceptibility of *C. albicans* towards chitinolytic bacteria. This may be ascribed to the fact that the mannan layer of *C. albicans*, with its low permeability, remained unchanged when the yeast is transferred from an aerobic to an anaerobic environment. Also, secondary metabolite production seems to be inhibited under anaerobic conditions. Interestingly, antifungal compounds, other than prodigiosin, that may be produced by some representatives of the bacterial isolates we studied, such as bacillomycin known to be produced by *B. subtilis* (Landy *et al*., 1948; Roongsawang *et al*., 2002; Swinburne *et al*., 1975), and biosurfactants known to be produced by *Enterobacter* (Jadhav *et al*., 2011), are all optimally produced under aerobic conditions.

Our results indicate that the synergistic effect of bacterial hydrolytic enzymes, capable of degrading different components of fungal cell walls, and antifungal agents may only function optimally under aerobic conditions. This might explain why *C. albicans* would be able to survive in oxygen limited aquatic zones (Stone *et al*., 2012), but not in aerobic zones found in the external environment away from an animal host. In addition, we found that bacterial mannan degrading enzymes may have a greater synergistic effect with an antifungal compound than chitinases in controlling *C. albicans* growth. To obtain a more comprehensive understanding of these reactions and the yeast-bacterial symbioses involved in the natural environment, it is envisaged that the interactions between *C. albicans* and bacterial communities are studied by analysing the total bacterial secretome, as well as the yeast proteome under aerobic and anaerobic conditions.

It is generally believed that the gastrointestinal (GI) tract of humans is the main reservoir for *C. albicans* (Nucci and Anaissie, 2001), and it is known that most regions of the GI tract are considered to be hypoxic to anaerobic (He *et al*., 1999). Thus, our findings potentially contribute to current knowledge on growth and survival of *C. albicans* in the presence of bacteria within its human host. Experiments aimed at studying interactions between *C. albicans* and human GI bacteria, under conditions simulating the anaerobic conditions of the GI tract, are expedient.

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Chapter 3

Binary Interactions of Bacteria with *Candida albicans* at 37ºC

Binary Interactions of Bacteria with *Candida albicans* at 37ºC

3.1 Introduction

It is known that *Candida albicans* forms part of the normal microbiome of healthy individuals (Chester and Cooper 2011). When the immune system is compromised however, this yeast acts as an opportunistic pathogen by colonizing and invading host tissue. Since it can survive under both aerobic and anaerobic conditions (Moran et al. 2012), this yeast is able to colonize a broad range of niches within the human host, but it also exposes *C. albicans* to a greater diversity of bacteria. It is thought that the numbers of this pathogen are kept in check within its human host by surrounding commensal bacteria (Boris and Barbés 2000; Kennedy and Volz 1985). The bacterial members of the microbiome not only compete with *C. albicans* for adhesion sites and nutrients, but they also secrete antimicrobials that suppress yeast growth as well as substances that may reduce the ability of this yeast to attach to certain mucosal structures (Boris and Barbés 2000; Kennedy and Volz 1985).

Thanks to the human microbiome project (Turnbaugh et al. 2007), our knowledge regarding different types of human commensal microorganisms increased extensively. It is known that on average the microbiome of the human gastrointestinal (GI) tract can consist of up to 5000 bacterial taxa (Dethlefsen et al. 2008). Therefore, it will be an enormous task to determine the role each bacterial species, or even genus, plays in controlling growth of *C. albicans* in the human GI tract, especially since a large number of these bacteria remain unculturable (Human Microbiome Jumpstart Reference Strains Consortium 2010). This is further complicated by unknown variables that may affect the numbers of this pathogen within its human host.

One such unknown variable is the effect of neighbouring bacteria on candidal growth in the anaerobic parts of the human GI tract. In chapter 2 we demonstrated that growth of *C. albicans* is inhibited by several bacteria under aerobic conditions at 26ºC, but this effect is ameliorated under anaerobic conditions. It must be noted however, that even though it is known that most regions of the human GI tract are hypoxic to anaerobic (He et al. 1999) and that *C. albicans* occurs in its yeast form under these conditions (White et al. 2007), microbial trials were mostly conducted with the infectious hyphal form of this yeast that is being

aerobically incubated at 37ºC (Boon et al. 2008; Hogan and Kolter 2002; Gibson et al. 2009; Peleg et al. 2008; Peleg et al. 2010; Tampakakis et al. 2009; Wang et al. 2004). To date, not many studies focused on the survival of *C. albicans* in its yeast form while incubated under anaerobic conditions.

A study conducted by Dumitru et al. (2004) however, revealed that under anaerobic conditions *C. albicans* seems to be less sensitive to certain antifungals than under aerobic conditions. This may contribute to the persistence of *C. albicans* in the human GI tract and the difficulty in completely eradicating this pathogen. To our knowledge no follow-up experimentation was done during the past decade to further explore the reasons for *C. albicans* being more resistant to antifungals under anaerobic conditions.

In chapter 2 we reported that under aerobic conditions, mannanases and the antimicrobial compound, prodigiosin, act synergistically to eradicate *C. albicans* at 26ºC. These findings could be ascribed to degradation of the outer mannan layer of the yeast's cell wall known to have low permeability and porosity, which would increase the susceptibility of the cell to antimicrobials such as prodigiosin and antifungals (Gow and Hube 2012). Whether a similar synergistic effect occurs for anaerobic *C. albicans* cells cultivated at 37ºC is not yet known. Considering the numbers of bacteria present in the human GI tract (Dethlefsen et al. 2008), the possibility of a synergistic effect between bacterial extracellular enzymes and antimicrobials within the GI tract is not unlikely. From all known phyla present in the human GI tract however, representatives of only one, i.e. Bacteroidetes are known to possess enzymes able to completely degrade the α-mannan present on the outer layer of yeast cell walls (El Kaoutari et al. 2013).

Keeping the above in mind, we hypothesized that at 37ºC under anaerobic conditions *C. albicans* would be more resistant to negative interactions, compared to under aerobic conditions at 37ºC. These interactions include antagonistic interactions with bacterial species known to occur in the human GI tract, antimicrobials such as prodigiosin and the antifungal agent Amphotericin B, as well as the actions of cell wall degrading α-mannanases originating from bacteria. To test this hypothesis we studied binary interactions at 37ºC between strains of *C. albicans* and selected bacteria known to occur in the human GI tract, under both anaerobic and aerobic conditions. Additionally, we screened the bacteria for chitinase and mannan degrading enzymes, under both anaerobic and aerobic conditions. Also, we determined the minimum inhibitory concentration of Amphotericin B using the reference

broth macrodilution susceptibility test, under both anaerobic and aerobic conditions. We then proceeded to test whether the presence of cell wall degrading enzymes, would enhance the antifungal activity of Amphotericin B and prodigiosin against unicellular anaerobically cultivated *C. albicans*.

3.2 Material and Methods

3.2.1 Strains used in this study

Clinical yeast strains, i.e. *Candida albicans* MRC 8908 and *Candida albicans* MRC 8912 were obtained from Tygerberg Hospital, Cape Town, South Africa. The yeasts *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 that were used as control strains to determine the minimum inhibitory concentration (MIC) of Amphotericin B, according to the CLSI M27-3A document (CLSI, 2008), were purchased from American Type Culture Collection. All yeast strains were stored in Yeast Malt Extract (YM) (Yarrow, 1998) broth supplemented with 30% (w/v) glycerol at -80°C and on YM agar slants.

The bacterial strains that were used in the experimentation are listed in Table 3.1. *Bacteroides* strains were purchased from the American Type Culture Collection and stored in brain heart infusion (BHI) broth (Biolab Diagnostics, Midrand, South Africa) supplemented with 30% (w/v) glycerol at -80°C and on agar slants containing BHI. *Escherichia coli* ATCC 13706 was purchased from the American Type Culture Collection, while *Klebsiella pneumoniae* CAB 1101 (Benadé et al. 2015) and *Clostridium perfringens* CAB 95 were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa and stored in Luria broth (LB) supplemented with 30% (w/v) glycerol at -80°C as well as on LB agar slants.

The two *Lactobacillus* strains (Table 3.1) were isolated from a commercially available nine strain probiotic supplement. One capsule was added to 9 ml physiological saline solution (PSS; 0.89% (w/v) NaCl), here after a dilution series was performed and plated out on BHI agar and plates were incubated for 48 h at 37ºC under anaerobic conditions. Two bacterial colonies were randomly selected and purified by repetitive subculturing on BHI agar. To identify these two bacterial strains, genomic DNA was first extracted from each isolate by using the ZR Fungal/Bacterial DNA MiniPrep™ system (Zymo Research, California, USA)

according to the manufacturer's instructions. Thereafter, 16S (1500 bp) region of the 30S small subunit ribosomal RNA was amplified by polymerase chain reaction (PCR) using universal ribosomal 16S primers (FWD 5′-AGTTTGATCCTGGCTCAG-3′ REV 5′-TACCTTGTTACGACTTCACCCCA-3), in an Applied Biosystems 2027 Thermal cycler (California, USA) The reaction consisted of 10 µl PCR master mix (2X) (Thermo Scientific, South Africa), 0.8 µl of each primer (Inqaba Biotechnical Industries, Pretoria, RSA), 1 µl DNA and 7.4 µl ddH_2O (double distilled water). The thermal cycling parameters were an initial denaturation at 95ºC for 1 min, followed by 30 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s, extension at 72ºC for 30 s, and final extension at 72ºC for 7 min (Benadé et al. 2015). The PCR products were visualised under UV light (Geneflash Syngene Bioimaging Unit, Cambridge, UK) after electrophoresis in a 0.8 % (w/v) ethidium bromide containing agarose gel (Horizon 11.4 GIBCO DRL Horizontal Gel Electrophoresis Apparatus, Life Technologies, California, USA). Subsequently, PCR products were purified with Nucleospin® Extract II chromatography columns (Macherey-Nagel, Duren, Germany). Samples were sequenced using an ABI PRISM (model 3100) genetic sequencer (Applied Biosystems, California, USA). The sequences were compared to reported sequences using a BLAST search on the NCBI GenBank database (Altschul et al. 1990). Only sequences with ≥ 99% homology to known sequences were regarded as meaningful for identification to species level.

3.2.2 Media, culture conditions and starter inoculums

For all co-culture experimentation, a quarter strength BHI broth (9.25 g/l) was used as growth medium, and the cultures were incubated for 48 h at 37ºC. Co-cultures containing anaerobic bacteria were incubated under anaerobic conditions without shaking, while co-cultures containing facultative anaerobic bacteria were incubated under both aerobic and anaerobic conditions. In each case, anaerobic conditions were obtained with anaerobic chambers containing Anaerocult A sets (Merck, Germany), and were monitored with Anaerotest strips (Merck, Germany). Anaerobic yeast inocula were prepared by inoculating test tubes containing 5 ml YM broth and incubating it for 48 h at 37ºC under anaerobic conditions. Aerobic yeast inocula were incubated for 24 h at 30ºC. In each case cells were subsequently harvested via centrifugation (12 000 × *g*, 5 min) in 2 ml microcentrifuge tubes. Each resulting pellet was washed three times with PSS. Yeast cells were subsequently resuspended in PSS,

enumerated using a haemocytometer (Improved Neubauer, Marienfeld Superior, Germany), and then each suspension was diluted with PSS to produce the desired concentration. Bacterial inocula were prepared in the same manner, however BHI broth was used as growth medium and the cells were enumerated using a Petroff-Hausser counting chamber (Improved Neubauer, Marienfeld Superior, Germany). Unless otherwise specified, all yeast and bacterial inocula were prepared as described above.

3.2.3 Co-cultures

Both *C. albicans* strains together with seven bacterial strains were used in the anaerobic experimentation (Table 3.1 and 3.2). Triplicate co-culture assays were conducted in test tubes, containing a quarter strength BHI broth, each inoculated with log 6 yeast cells/ml and log 6 bacterial cells/ml. After 48 h of anaerobic incubation in the dark at 37ºC, yeast cells were enumerated using dilution plates prepared with YM agar supplemented with 200 mg/l chloramphenicol (Sigma-Aldrich, Steinheim, Germany), and incubated aerobically at 30ºC. The bacteria in each co-culture were enumerated using dilution plates prepared with BHI medium that were incubated for 48 h at 37ºC. Plates of both *Bacteroide*s strains and *C. perfringes* were incubated under anaerobic conditions, while the two *Lactobacillus*, *E. coli* and *K. pneumoniae* plates were incubated under aerobic conditions. Yeast and bacterial monocultures were included as controls in each case.

Aerobic triplicate co-culture assays were prepared in the same manner as described for the anaerobic co-cultures; however, after 48 h of incubating them on a tissue roller drum (8 rev/min) in the dark at 37ºC, yeast viability was determined by staining yeast cells with the LIVE/DEAD® FungaLight™ Yeast Viability Kit (Invitrogen Molecular probes) according to the manufacturer's instructions. Wet mounts were subsequently prepared from the stained cells that were then visualized with a Nikon Eclipse E400 epifluorescence microscope, equipped with a multipass filter set appropriate for viewing 40,6-diamidino-2-phenylindole (DAPI), as well as excitation/barrier filter sets of 465–495/515–555 and 540–580/600–660 nm. Images were captured with a Nikon DS-Fi2 camera and a Nikon Digital Sight DS-U3 camera controller (Nikon, Japan). Bacteria from the aerobic co-cultures were enumerated as described previously.

3.2.4 Plate assays for α-mannanase and chitinase activity

Accept for the anaerobic bacteria used in the experimentation, all bacterial strains were aerobically and anaerobically screened for extracellular hydrolytic enzyme while growing on agar media. In each case a sterile pipet tip was used to spot inoculate the middle of an agar plate, thereafter the plates were incubated at 37ºC for 72 h. Mannanase activity was tested according to Benadé et al. (2015). Briefly, medium containing 1.0% (w/v) mannan from *C. albicans* CAB 1084, 0.67% (w/v) filter sterilized Yeast Nitrogen Base, pH adjusted to 7 (Difco, YNB), and 2.0% (w/v) bacteriological agar was used to test for mannanase activity. The mannan used in the medium was obtained via alkali extraction using a method adapted from literature (Castro et al. 1995; Gacto et al. 2000). Mannanase activity was revealed by clear halos around the bacterial colonies. Chitinase production was examined using agar plates containing 0.5% (w/v) colloidal chitin (Ahmadi et al. 2008) prepared with chitin from shrimp shells (Sigma-Aldrich, Steinheim, Germany), 0.065% (w/v) Na_2HPO_4, 0.15% (w/v) KH_2PO_4, 0.025% (w/v) NaCl 0.05% (w/v) NH_4Cl, 0.012% (w/v) $MgSO_4$ and 0.0005% (w/v) $CaCl_2$. Chitinase production was revealed by clear halos around bacterial colonies growing on an otherwise opaque medium.

3.2.5 Determining minimum inhibitory concentration (MIC) of Amphotericin B using the reference broth macrodilution susceptibility test

Amphotericin B (AmB) was obtained from Merck (Germany). Stock solutions of the antifungal agent was prepared and stored frozen at -20ºC until use. Broth macrodilution susceptibility tests were performed in accordance with CLSI M27-3A standards. Briefly, serial twofold dilutions of AmB ranging from 0.313 to 160 µg/ml was prepared in RPMI 1640 containing L-glutamine without bicarbonate (Sigma-Aldrich, Steinheim Germany) which was buffered to pH 7.0 with 0.165 M MOPS (morpholinepropanesulfonic acid) (Sigma-Aldrich, Steinheim Germany). Amphotericin B stock solution (1600µg/ml) was dissolved in dimethyl sulfoxide (DMSO), thereafter a dilution series was prepared using DMSO that yielded a concentration of 3.313 to 1600 µg/ml. The dilutions were subsequently further diluted tenfold in RPMI 1640 broth, which resulted in a working concentration of 0.313 to 160 µg/ml. *Candida albicans* MRC 8908 and MRC 8912 as well as the two control

strains, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were grown on Sabouraud glucose agar (SGA) plates for 18 to 24 h at 37°C under aerobic conditions. Five colonies of each yeast strain were used to prepare yeast cell suspensions in PSS. Thereafter, yeast cell densities were adjusted after determining the concentration with a haemocytometer to yield yeast stock suspensions of 3.0 x 10^6 cells/ml. Subsequently, the stock suspensions were diluted 1:100 followed by 1:20 dilution that yielded working suspensions of 1.5 x 10^3 cells/ml. Here after, 0.1 ml of each AmB concentration was added to a test tube. To each of these test tubes, 0.9 ml of the yeast stock suspension was added. This resulted in a 1:10 dilution of the antifungal concentration and a 10% dilution of the yeast inoculum. The control tube contained RPMI 1640 broth and a yeast cell suspension with no antifungal agent. The tubes were incubated at 35°C (without shaking) under aerobic conditions and results were read after 24 and 48 h. This experimentation was also conducted under anaerobic conditions. The MICs were calculated for AmB under aerobic and anaerobic conditions.

3.2.6 Determining MIC using cell counts

The broth macrodilution susceptibility tests were repeated as described above, however tubes were incubated under both aerobic and anaerobic conditions. Subsequently, yeast cells were enumerated after 5 and 48 h at 37ºC using dilution plates prepared with YM agar supplemented with 200 mg/l chloramphenicol, and incubated aerobically at 30ºC.

3.2.7 Effect of AmB or prodigiosin on *C. albicans* strains in the presence of extracellular enzymes originating from bacterial strains

An inoculum of *C. albicans* was prepared as described in the previous sections. Crude preparations of extracellular enzymes originating from *K. pneumoniae* CAB 1101 and *B. fragilis* NCTC 9343 were prepared by growing each bacterial strain in 250 ml conical flasks containing 25 ml LB or BHI medium respectively. A flask containing *K. pneumoniae* CAB 1101 was incubated at 37ºC for 48 h under aerobic conditions, while the flask containing *B. fragilis* NCTC 9343 was incubated at 37°C for 48 h under anaerobic conditions. Supernatants of each culture were obtained via centrifugation (15 000 × *g*, 10 min), and these supernatants were dialyzed at 4ºC for 24 h against 50 mM $NaPO_4$ (pH 7.0) using molecular porous dialysis

tubing (Spectra/por 1 dialysis cellulose membrane, molecular weight cut off 6 000 - 8 000 Da, Spectrum Medical Industries Inc., Houston, TX). In each case an aliquot of 1.2 ml of the resulting crude enzyme preparation, was transferred to a 2 ml microcentrifuge tube. To test the synergistic effect of AmB or prodigiosin and bacterial extracellular mannanases on yeast cells, anaerobically cultivated washed cells of *C. albicans* 8908 was subsequently suspended in each of the crude enzyme preparations to reach a final concentration of log 3.13 yeast cells/ml for AmB experimentation, and log 6 yeast cells/ml for prodigiosin experimentation. The suspensions were incubated at 37°C (without shaking) for a total of 2 h. After the first hour of incubation AmB (final concentration of 0.06 µg/ml) or prodigiosin (final concentration 100 µg/ml) was added to each of these suspensions. Plate counts, to enumerate culturable yeasts, were subsequently conducted using YM plates incubated at 30°C. All experiments described above contained negative controls in which the crude enzyme extracts were inactivated by 15 min of boiling. All experiments were conducted in triplicate. To confirm the presence or absence of mannanase in both active and inactivated crude enzyme preparations used to treat living cell suspensions of *C. albicans* 8908, 500 µl of each crude enzyme preparation was dripped onto mannan plates. These plates were incubated at 37ºC for 16 to 72 h while being monitored for clear zones on the opaque agar where the filtrate was dripped onto.

3.3 Results

3.3.1 *Candida albicans* in the presence of different bacteria under anaerobic conditions

The survival of the two *C. albicans* strains after 48 h at 37ºC under anaerobic conditions, in co-cultures prepared separately with each of the seven anaerobic or facultative anaerobic bacterial strains is depicted in Fig. 3.1. None of the bacterial strains seemed to influence the proliferation of *C. albicans*. In both cases, yeast numbers in the presence of a bacterial strain did not significantly differ from the yeast numbers of the *C. albicans* monoculture.

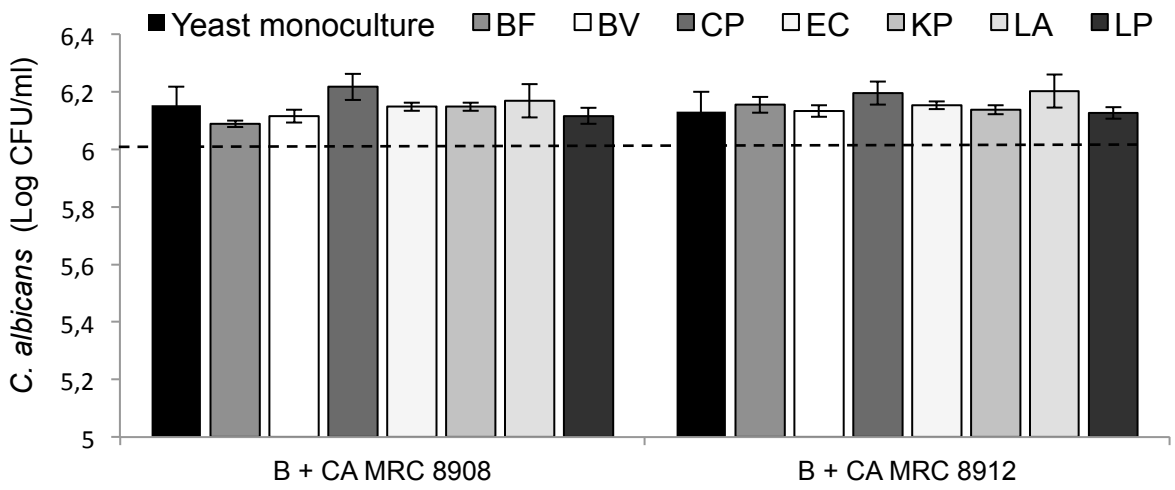


Fig. 3.1 Concentrations of *Candida albicans* (CA) MRC 8908 and MRC 8912 when co-cultured separately with each of the seven bacterial isolates for 48 h at 37ºC under anaerobic conditions. B, bacterial strain; BF, *Bacteroides fragilis* ATCC 9343; BV *Bacteroides vulgatus* ATCC 8482; CP, *Clostridium perfringens* CAB 95; EC. *Escherichia coli* ATCC 13706; KP, *Klebsiella pneumoniae* CAB 1101; LA, *Lactobacillus acidophilus* CAB 106; LP *Lactobacillus plantarum* CAB 105. In each case the first bar represents the control, *C. albicans* monoculture. Bars represent the mean of three repetitions and the whiskers indicate the standard error. The dashed line represents the initial concentration of the yeasts. None of the co-culture yeast concentrations was significantly less than the control (Fisher LSD test, $p > 0.05$; Statistica Version 13, StatSoft).

3.3.2 Bacteria in the presence of the two *C. albicans* strains under anaerobic conditions

Bacterial cell concentrations of the seven anaerobic and facultative anaerobic bacteria, after 48 h at 37ºC under anaerobic conditions, in co-cultures prepared with *C. albicans* MRC 8908 and MRC 8912 are depicted in Fig. 3.2. Except for the two *Bacteroides* strains, bacterial growth seemed to be unaffected by the presence of the yeast. Both *B. fragilis* ATCC 9343 and *B. vulgatus* ATCC 8482 numbers are significantly enhanced in the presence of both *C. albicans* strains.

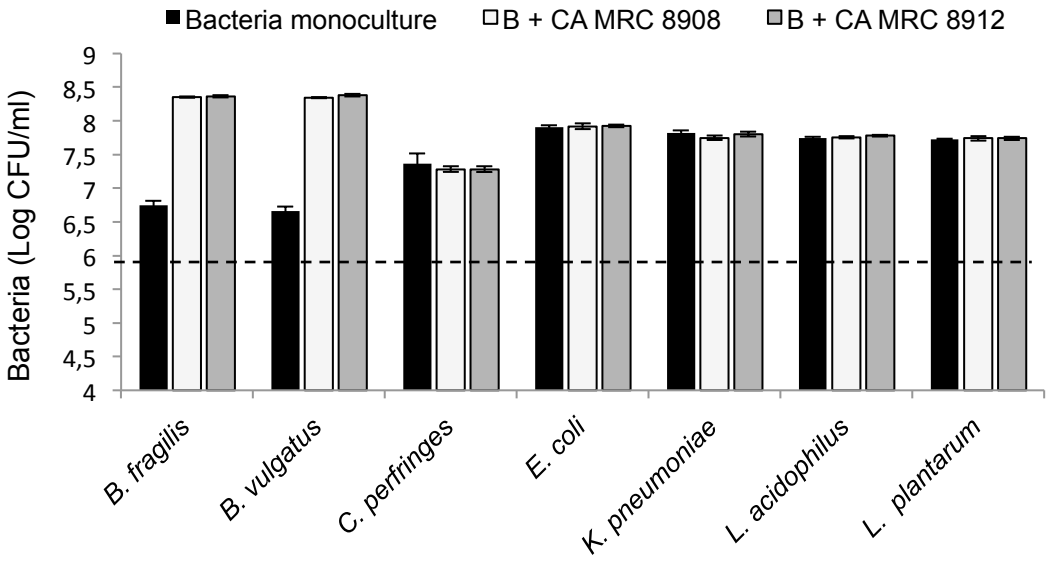
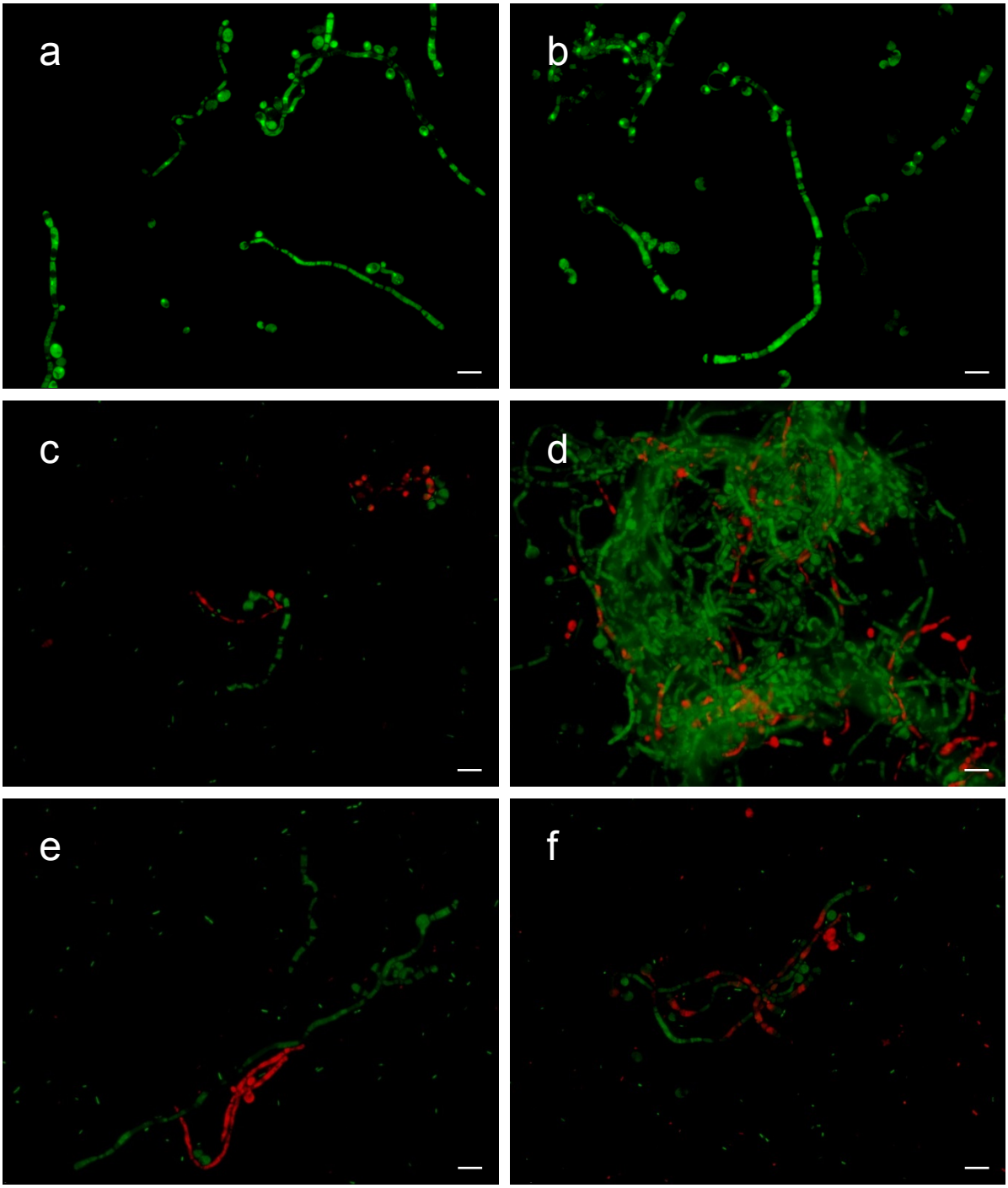


Fig. 3.2 Concentrations of the seven bacterial (B) strains when co-cultured separately with the two *C. albicans* (CA) strains for 48 h at 37ºC under anaerobic conditions. In each case the first bar represents the control, bacterial monoculture. Bars represent the mean of three repetitions and the whiskers indicate the standard error. The dashed line represents the initial concentration of the yeasts. The co-cultured *Bacteroides* numbers were significantly higher than that of the control in both cases (Fisher LSD test, $p \leq 0.05$; Statistica Version 13, StatSoft), while none of the other co-culture bacterial concentrations was significantly different than the monocultures (Fisher LSD test, $p > 0.05$.).

3.3.3 Live/dead staining of *C. albicans* MRC 8908 after aerobic co-culturing

Photomicrographs of *C. albicans* when co-cultured with the four different facultative anaerobic bacteria after 48 h at 37ºC under aerobic conditions are depicted in Fig. 3.3. Cells from the *C. albicans* monoculture almost always stained green, indicating live cells (Fig. 3a and b). These cells were mostly in hyphal phase. When this yeast was co-cultured with *Escherichia coli* ATCC 13706, some dead cells were visible (stained red) (Fig. 3c-f); however, whether there was significantly fewer live cells compared to the control, was not determined. In the co-cultures containing *K. pneumoniae* CAB 1101, it seems that most yeast cells which stained red were enclosed with bacterial cells (Fig. 3g-j). Interestingly, *C. albicans* co-cultured with the two *Lactobacillus* species did not form hyphae and stained mostly green, indicating live cells (Fig. 3k-n). In all cases bacterial numbers in the presence

of *C. albicans* did not significantly differ from the bacterial numbers of their monocultures (results not shown).



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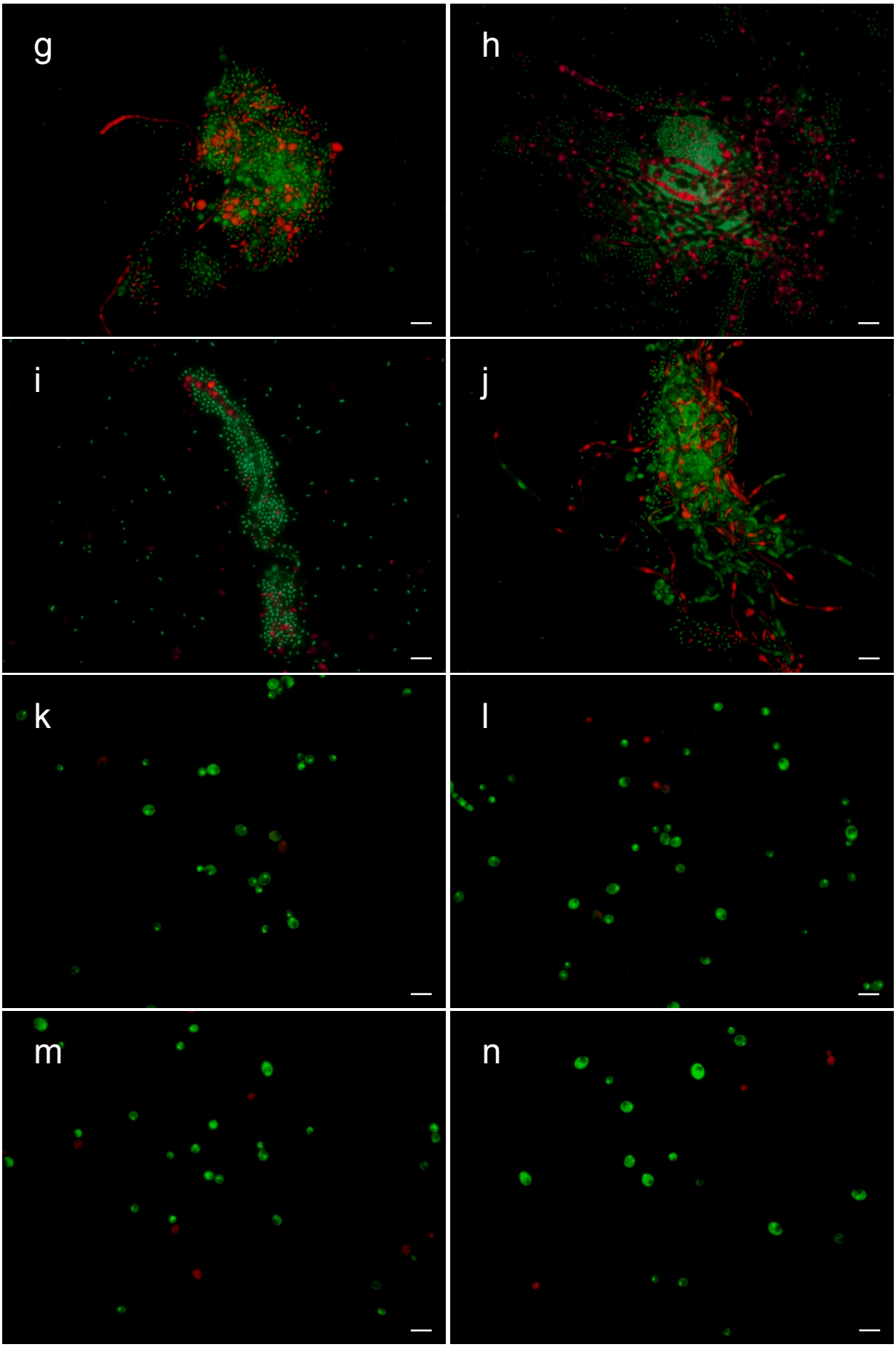

Fig. 3.3 Photomicrographs of LIVE/DEAD® -stained *Candida albicans* MRC 8908 cells. Live cells stained green, while dead cells stained red. **a, b** *C. albicans* cells from the monoculture after 48 h at 37ºC under aerobic conditions. **c-f** *C. albicans* when co-cultured with *Escherichia coli* ATCC 13706 after 48 h at 37ºC under aerobic conditions. **g-j** *C. albicans* when co-cultured with *Klebsiella pneumoniae* CAB 1101 after 48 h at 37ºC under aerobic conditions. It is clearly noticeable how the bacterial cells are packed around *C. albicans*. **k, l** *C. albicans* when co-cultured with *Lactobacillus acidophilus* CAB 106 and *Lactobacillus plantarum* CAB 105 **(m, n)** after 48 h at 37ºC under aerobic conditions. *Candida albicans* did not form hyphae in the presence of either *Lactobacillus* species. Scale bars represent 10 μm.

3.3.4 Extracellular chitinase and mannanase production

None of the anaerobic or facultative anaerobic bacteria showed chitinase activity at 37ºC. The two *Bacteroides* strains showed mannanase activity under anaerobic conditions, while *K. pneumoniae* CAB 1101 tested positive for mannanase under aerobic conditions (Table 3.1). *Clostridium perfringens* CAB 95 tested negative for mannanase under anaerobic conditions, while all the facultative anaerobic bacteria, except for *K. pneumoniae* CAB 1101, showed no mannanase activity under both aerobic and anaerobic conditions.

Table 3.1 The oxygen requirements and α-mannanase activity of bacterial strains used in this study.

Bacterial strains	Oxygen requirements	α-mannanse activity
Bacteroides fragilis ATCC 9343	Anaerobe	+ *
Bacteroides vulgatus ATCC 8482	Anaerobe	+ *
Clostridium perfringens CAB 95	Anaerobe	-
Escherichia coli ATCC 13706	Facultative anaerobe	-
Klebsiella pneumoniae CAB 1101	Facultative anaerobe	+ #
Lactobacillus acidophilus CAB 106	Facultative anaerobe	-
Lactobacillus plantarum CAB 105	Facultative anaerobe	-

+, Positive for enzyme activity; -, Negative for enzyme activity, * Detected under anaerobic conditions; # Detected under aerobic conditions

3.3.5 MIC of AmB against *C. albicans* under aerobic and anaerobic conditions

The MICs that were determined using the reference broth macrodilution susceptibility test are shown in Table 3.2. The aerobic MIC for both *C. albicans* strains as determined with the macrodilution test, is 0.06 µg/ml. Using the macrodilution test, the anaerobic MIC could not be determined since turbidity of the yeast in the RPMI 1640 broth did not reach a point of visibility. Therefore, yeast cell counts were conducted after 5 and 48 h at 37ºC to compare aerobic and anaerobic MICs (Fig. 3.4). The MIC determined after 48 h using cells counts, was 0.06 µg/ml for both yeast strains under aerobic and anaerobic conditions.

Table 3.2 The MIC of Amphotericin B, as determined with the reference broth macrodilution and plate count methods, for the two yeast strains used in this study.

***Candida* strains**	**MICs obtained with macro brothdilution test (µg/ml)**		**MICs obtained with cell counts (µg/ml)**	
	Aer	An	Aer	An
CA MRC 8908	0.06	ND	0.06	0.06
CA MRC 8912	0.06	ND	0.06	0.06

CA: *Candida albicans*; MIC: minimum inhibitory concentration of AmB; ND: Not determined

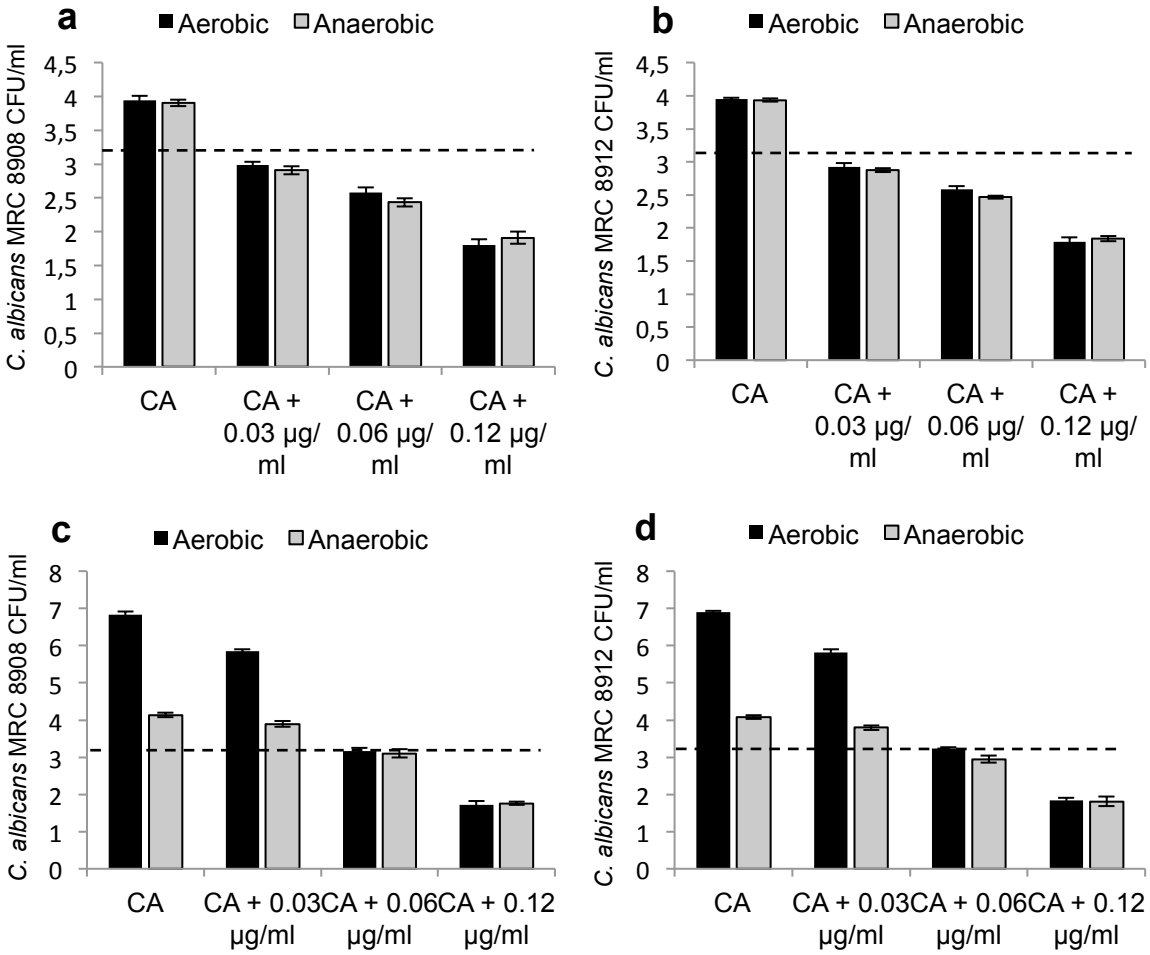

Fig. 3.4 Concentration of, **a** *Candida albicans* (CA) MRC 8908 and **b** *C. albicans* (CA) MRC 8912 after 5 h in the presence of different concentrations of AmB at 37ºC under both aerobic and anaerobic conditions, as well as the concentration of **c** *C. albicans* MRC 8908, and **d** *C. albicans* MRC 8912 after 48 h in the presence of different concentrations of AmB at 37ºC under both aerobic and anaerobic conditions. In each case the first two bars represent the control tubes without any AmB. Bars represent the mean of three repetitions and the whiskers indicate standard error. The dashed line represents the initial concentration of the yeast. There were no significant differences in the yeast concentrations of the aerobic and anaerobic cultures after 5 h of incubations at 37ºC (Fisher LSD test, $p > 0.05$; Statistica Version 13, StatSoft). After 48 h incubation at 37ºC, anaerobic *C. albicans* concentrations of both the control tubes and tubes containing 0.03 µg/ml AmB were significantly less than the aerobic yeast concentrations (Fisher LSD test, $p \leq 0.05$). However, there was no significant difference between aerobic and anaerobic yeast concentration in the presence of 0.06 and 0.12 µg/ml

AmB (Fisher LSD test, $p > 0.05$). The MIC for both aerobic and anaerobic cells is 0.06 µg/ml AmB.

3.3.6 Fate of *C. albicans* in the presence of α-mannanase and AmB or prodigiosin

Figure 3.5 depicts the survival of *C. albicans* MRC 8908 in each of the crude extracellular enzyme preparations, originating from *B. fragilis* ATCC 9343 and *K. pneumoniae* CAB 1101. The crude enzyme preparations did not have a significant negative effect on the cell numbers of *C. albicans* MRC 8908. In contrast, both 0.06 µg/ml AmB and 100 µg/ml prodigiosin, showed a negative effect on yeast numbers in the presence of inactivated enzyme extracts. This negative effect was not synergistic with the action of the active enzyme extract from *B. fragilis* ATCC 9343 or *K. pneumoniae* CAB 1101. Both active crude enzyme preparation of *B. fragilis* ATCC 9343 and *K. pneumoniae* CAB 1101 showed mannanase activity on mannan containing plates.

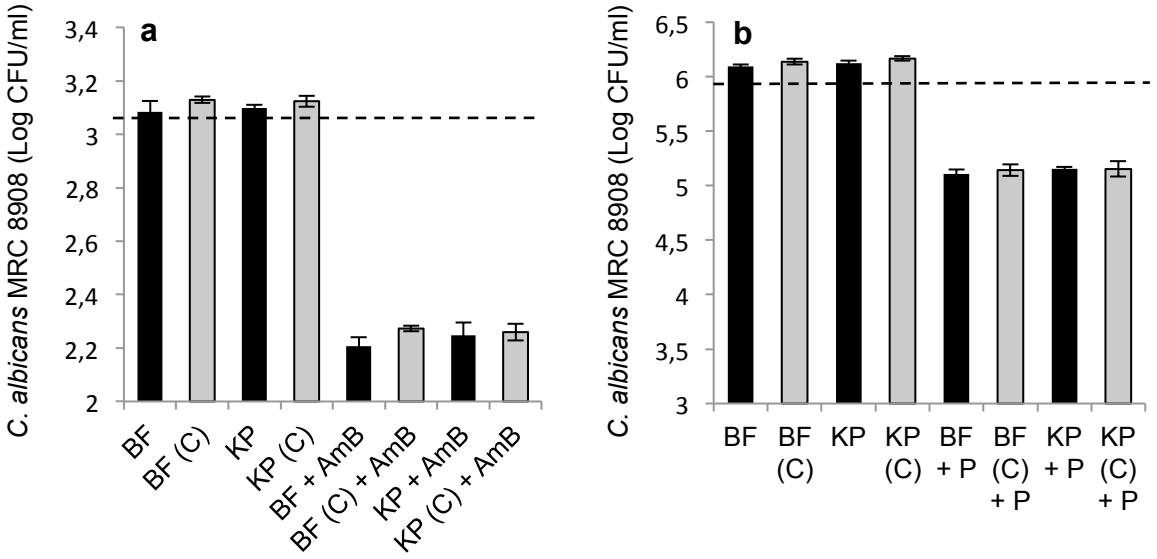

Fig. 3.5 Concentrations of culturable *Candida albicans* MRC 8908 suspended for 2 h in dialysed crude extracellular enzyme extracts of *Bacteroides fragilis* ATCC 9343 (BF) and *Klebsiella pneumoniae* CAB 1101 (KP), either in the absence or presence of (**a**) 0.06 µg/ml AmB or (**b**) 100 µg/ml prodigiosin (P). Inactivated enzyme extract represents the control (C) in each case. In both cases, there was no significant difference between the results obtained

with dialysed enzyme extracts and the inactivated dialysed controls (Fisher LSD test, $p > 0.05$; Statistica Version 13, StatSoft). Compared to these controls, significantly less culturable yeast cells were recovered from both the active and inactivated crude extracellular enzyme extracts when either 0.06 µg/ml AmB or 100 µg/ml prodigiosin was present in the yeast suspensions (Fisher LSD test, $p \leq 0.05$). These negative effects of AmB and prodigiosin on yeast cell numbers however, were not enhanced by the presence of the active enzyme extracts (Fisher LSD test, $p > 0.05$). Bars represent the mean of three repetitions and the whiskers indicate standard error. The dashed line represents the initial concentration of the yeast.

3.4 Discussion

Even though it has been known for some time that *C. albicans* in the human GI tract is kept in check by beneficial bacteria (Kennedy and Volz 1985), our results showed that seven bacterial strains, all occurring in the human GI tract, (Eggerth and Gagnon 1933; Finegold et al. 1974; Moore and Holdeman 1974; Rajilić-Stojanović et al. 2007; Rajilić-Stojanović 2007) did not have an effect on *C. albicans* proliferation under anaerobic conditions at 37ºC. The same phenomenon was found when *C. albicans* was co-cultured with different bacterial strains at 26ºC under anaerobic conditions (Chapter 2). In addition, similar to our findings as reported in chapter 2, most of the bacteria were unaffected by the presence of *C. albicans*, except for the two *Bacteroides* strains. The growth of both *B. fragilis* ATCC 9343 and *B. vulgatus* ATCC 8482 was significantly increased in the presence of *C. albicans* compared to the corresponding monoculture growth of the bacteria. A possible reason for this enhanced growth may be the capability of these bacteria to produce extracellular α-mannanases enabling them to utilize yeast cell wall mannans (El Kaoutari et al. 2013). It was previously been demonstrated that *Bacteroides* species are able to utilize yeast mannan originating from *C. albicans* and *Saccharomyces cerevisiae* (Cuskin et al. 2015).

Under aerobic conditions at 37ºC, *C. albicans* mostly grows in its hyphal phase (Sudbery et al. 2004) and therefore cell counts could not be used to study the effect of the four facultative anaerobic bacteria on this pathogen. Nevertheless, some dead yeast cells (stained red with LIVE/DEAD® staining; Fig. 3.3c-j) were visible when co-cultured with *E. coli* ATCC 13706 and *K. pneumoniae* CAB 1101, potentially indicating declining yeast numbers in the co-

cultures. This supported our results previously obtained (Chapter 2) with regard to the antagonistic effect of *K. pneumoniae* CAB 1101 on *C. albicans* at 26 ºC under aerobic conditions. We subsequently found that, similar to the results reported for enzyme production at 26ºC in chapter 2, this bacterial strain can produce α-mannanase at 37ºC (Table 3.1) enabling it to degrade yeast cell walls. Interestingly, it was clearly visible how *K. pneumoniae* cells associate with the *C. albicans* cells (Fig 3.3g-j). Furthermore, previous findings reported in literature also revealed how cells from the bacteria *Pseudomonas aeruginosa* and *Salmonella enterica* associate with the hyphae of *C. albicans* (Tampakakis et al. 2009; Hogan and Kolter 2002). In the co-cultures containing *C. albicans* and either *L. acidophilus* CAB 106 or *L. plantarum* CAB 105, the yeast did not grow in its hyphal form (Fig. 3.3k-n). This phenomenon has also been described in literature, where representatives of both *Burkholderia cepacia* (Boon et al. 2008) and *Xanthomonas campestris* (Wang et al. 2004) were found to inhibit the dimorphic transition of *C. albicans*. Similarly, Noverr and Huffnagle (2004) demonstrated the ability of *Lactobacillus rhamnosus* to inhibit germ tube formation of *C. albicans*.

Contrary to the findings of Dumitru et al. (2004), we did not find *C. albicans* to be more resistant to AmB under anaerobic conditions. These researchers used a starter inoculum of log 6 yeast cells/ml which is notably higher than the concentration (5.0×10^2 to 2.5×10^3 cells/ml) recommended by the CLSI M27-3A standards. This might possibly have had a significant effect on their findings. In our study, using lower starter inoculums, turbidity of the yeast in the RPMI 1640 broth in the anaerobic tubes, did not reach a point of visibility to determine the MIC of AmB. Hence, plate counts were performed to evaluate MIC under both aerobic and anaerobic conditions. Both *C. albicans* strains were found to have a MIC of 0.06 µg/ml AmB under both aerobic and anaerobic conditions. Similar results were obtained in a previous study (Hawser and Islam 1999).

In contrast to our previous findings on the synergistic effect of antimicrobials in combinations with cell wall degrading enzymes at 26ºC under aerobic conditions (Chapter 2), we found that at 37ºC the antagonistic effect of AmB on anaerobically cultured yeast was not enhanced in the presence of crude enzyme preparations (containing α-mannanase) originating from *B. fragilis* ATCC 9343 and *K. pneumonia* CAB 1101 (Fig. 3.5a). Similarly, when *C. albicans* cells were treated with prodigiosin in combination with crude enzyme preparations originating from *B. fragilis* ATCC 9343 and *K. pneumonia* CAB 1101, no synergistic effect

was observed. Consequently, either the temperature change or the fact that anaerobically cultured cells of *C. albicans* were used in the experimentation, may have contributed to this yeast being more resistant to the synergistic effect of prodigiosin and crude enzyme preparations containing α-mannanase.

In conclusion, our findings suggest that at 37ºC, *C. albicans* may be more resistant to some negative interactions under anaerobic conditions as compared to under aerobic conditions. These interactions include antagonistic interactions with some bacteria known to occur in the human GI tract. In contrast, *C. albicans* seems to be just as sensitive to the antifungal agent Amphotericin B under anaerobic conditions as compared to under aerobic conditions. In contrast to observations previously conducted under aerobic conditions at 26ºC (Chapter 2), the presence of bacterial cell wall degrading mannanases does not seem to enhance the antagonistic effect of antimicrobials such as Amphotericin B and prodigiosin. The role of yeast cell wall mannan in anaerobic interactions between bacteria and *C. albicans* will be studied further (Chapter 4).

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Chapter 4

Potential mannan-dependent symbiosis between human gut *Bacteroides* and the yeasts *Candida albicans* and *Saccharomyces cerevisiae*

Potential mannan-dependent symbiosis between human gut *Bacteroides* and the yeasts *Candida albicans* and *Saccharomyces cerevisiae*

4.1 Introduction

The two most common bacterial phyla in the gastrointestinal (GI) tract of healthy adult humans are the Firmicutes and Bacteroidetes (Eckburg et al. 2005; Zoetendal et al. 2008). Recently, a representative model human GI tract microbiome, which comprised of 177 bacterial genomes including 104 Firmicutes and 29 Bacteroidetes genomes, was studied for the distribution of carbohydrate digestive capacity among phyla (El Kaoutari et al. 2013). It was found that while representatives of both these phyla encoded for carbohydrate-active enzymes, individual representatives of Bacteroidetes generally encoded for more glycan-cleaving enzymes than individuals representing Firmicutes. Interestingly, some representatives of Bacteroidetes were also found to be the only bacteria encoding for a group of glycoside hydrolases (GH38, GH92 and GH76), able to completely degrade α-mannan (El Kaoutari et al. 2013) and known to occur on the exterior of yeast cell walls (Fig. 4.1a). These results supported previous findings which showed that the genome of some *Bacteroides* species may encode for up to 36 proteins associated with α-mannanase or α-mannosidase activity (Xu et al. 2003). More recently, detailed biochemical analyses and targeted gene disruption studies were conducted to show that some *Bacteroides* species are able to utilize yeast α-mannan originating from cell walls of *Candida albicans* and *Saccharomyces cerevisiae* (Cuskin et al. 2015). It was concluded that the ability of the *Bacteroides* species to utilize yeast mannan may be due to domestication of yeast and other fungi in the human diet. These studies therefore provided compelling evidence that *Bacteroides* species are capable of producing enzymes involved in degrading the cell walls of yeasts such as *S. cerevisiae* and *C. albicans*, both known to occur in the human GI tract (Moran et al. 2012; Pecquet et al. 1991). *Saccharomyces cerevisiae* may be transiently present in the human GI tract following oral ingestion (Pecquet et al. 1991; Scevola et al. 2013), while *C. albicans* generally occurs as a commensal in the human GI tract (Gow and Hube 2012; Moran et al. 2012). The latter yeast however, may also act as an opportunistic pathogen, although its population size is thought to be regulated by commensal bacteria (Kennedy and Volz 1985; Boris and Barbés 2000). The detrimental effect of bacteria on *C. albicans* cells was demonstrated for representatives of *Acinetobacter baumannii, Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Salmonella*

enterica, and *Xanthomonas campestris* (Tampakakis et al. 2009; Boon et al. 2008; Wang et al. 2004; Hogan and Kolter 2002; Peleg et al. 2008). It must be noted however, that these interactions were mostly studied using the hyphal form of this pleomorphic yeast, while being incubated under aerobic conditions at 37°C. Recently, it was found that growth of *C. albicans* in bacteria/yeast co-cultures at 26°C was inhibited by the presence of *Klebsiella pneumoniae* and *Serratia marcescens* under aerobic conditions. However, under anaerobic conditions no such inhibitory effect was observed and yeast and bacterial growth in co-cultures was found to be similar to that observed for pure cultures (Benadé et al. 2015). The inhibitory effect on yeast growth was ascribed to the aerobic production of bacterial antimicrobial compounds and bacterial extracellular hydrolytic enzymes, including chitinases and mannan degrading enzymes.

The above-mentioned findings showed that the external mannans in the cell wall of ascomycetous yeasts such as *C. albicans* and *S. cerevisiae* are subjected to hydrolysis by bacterial mannanases, like those known to be produced by some *Bacteroides* species. Whether the ability of *Bacteroides* to utilize the external cell wall layers of *C. albicans* and *S. cerevisiae* will have an impact on cellular interactions between these microbial taxa, remains unanswered. However, previous findings indicated increased survival of *Bacteroides* within *C. albicans* biofilms as a result of the anaerobic environment provided by the biofilm (Fox et al. 2014). The goal of this study was therefore to test the hypothesis that growth of *Bacteroides* strains, capable of degrading the outer mannan layers of ascomycetous yeasts, are enhanced by the presence of culturable cells of *C. albicans* or *S. cerevisiae*. To test this hypothesis we first studied binary interactions between representative yeast strains and representatives of *Bacteroides* known to produce α-mannan degrading enzymes. Then we determined the effect of the mannan layers in yeast cell walls on the *Bacteroides* strains by evaluating bacterial growth in the presence of engineered *S. cerevisiae* showing reduced outer mannan layers as a result of deletion mutations of mannosyltransferase genes (Ballou 1990). Finally, we evaluated growth of the *Bacteroides* strains on dead cells of *C. albicans* and *S. cerevisiae,* with and without the external mannan layer.

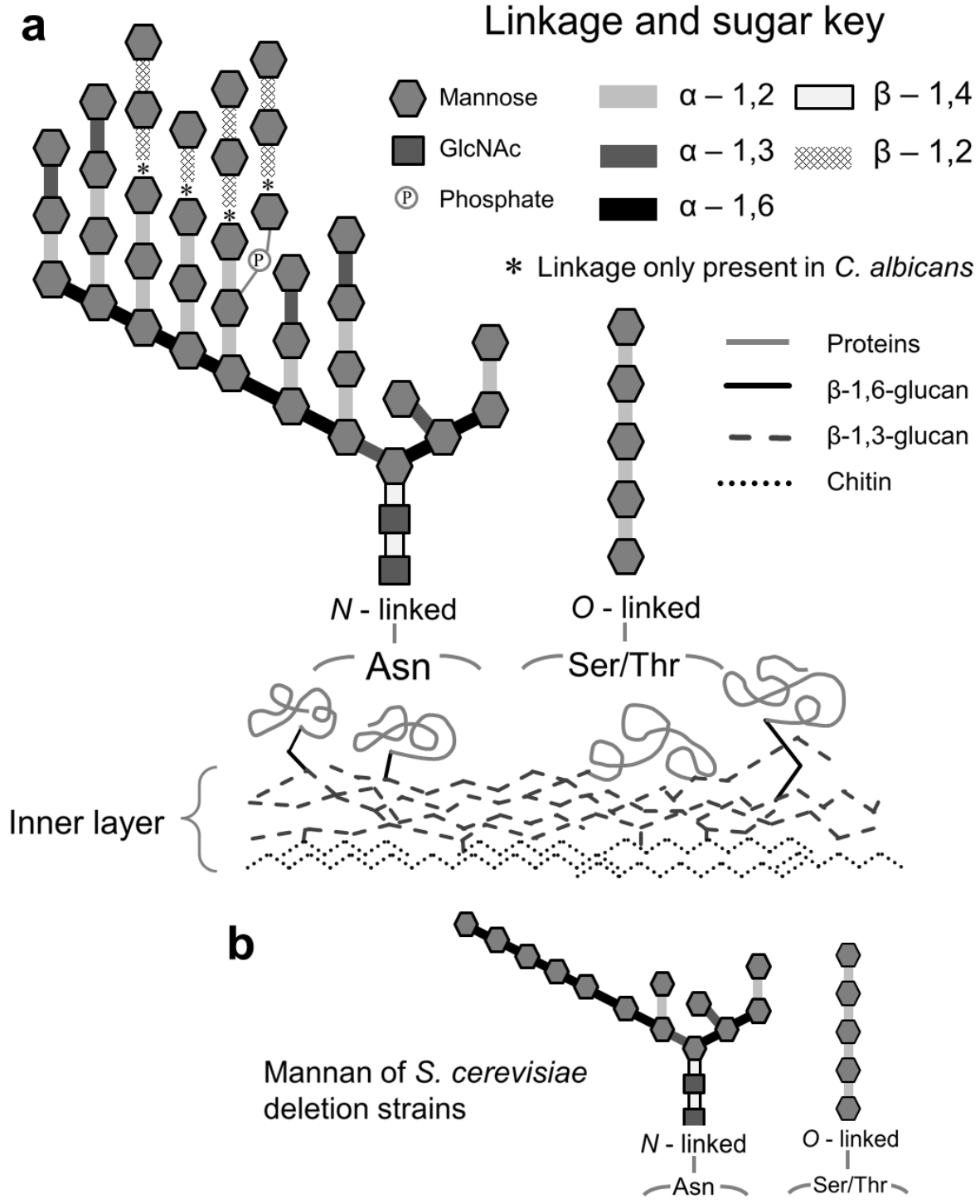

Fig. 4.1 a Schematic representation of the mannan layer, on the exterior of wild type *C. albicans* and *S. cerevisiae*, which can comprise up to 40 % (w/w) of the cell wall of ascomycetous yeasts (Klis 1994). The mannan layer is formed by mannoproteins that are extensively glycosylated in the ER lumen and Golgi apparatus before being transported to the cell wall. On average these glycosylated mannoproteins contain between 150 and 200

mannose residues (Munro 2001). Glycosylation in *S. cerevisiae* occurs via the action of multiple protein complexes responsible for the successive elongation of the poly-α-1,6-mannan backbone. The core structure of the N-glycan is formed through the activity of Alg1 (α-1,3-mannose transferase) and Och1 (α1,6-mannose transferase). The mannan polymerase I complex (contains Mnn9 and Van1 α1,6-mannosyltransferases) adds the first 10-15 α-1,6-mannose residues to the core structure, after which the mannan polymerase II complex (contains Mnn9, Anp1, Mnn10, Mnn11 and Hoc1 α-1,6-mannosyltransferases) elongate the α-1,6 mannan backbone with an additional 40-60 mannose residues. Subsequent activities by various transferases form the final branched and phosphorylated macromolecular structures that are linked to mannoproteins (Orlean 2012). These N-linked oligomannosides are linked to cell wall proteins by N-glycosidic bonds to asparagine (Asn) residues (Dean 1999). The only difference between the N-linked mannan structure of *C. albicans* and *S. cerevisiae* is that *C. albicans* contains β-1,2 oligomannosides on the end of the side chains. In addition, both *C. albicans* and *S. cerevisiae* mannan can also be attached to cell wall proteins by O-glycosidic bonds to threonine (Thr) or serine (Ser) residues. These O-linked oligomannosides are single or short unbranched residues joined through α-1,2 linkages and can vary in length from two to six mannose residues (Nelson et al. 1991; Strahl-Bolsinger et al. 1999). Only in *S. cerevisiae* can these O-mannan be capped by several α-1,3-mannose units (Orlean 2012). **b** The compromised mannan layer of *S. cerevisiae mnn10* and *mnn11* deletion strains, consisting only of short poly-α1,6-mannan backbones attached to their mannoproteins, as well as the short chain O-linked mannans also occurring in wild type strains.

4.2 Material and Methods

4.2.1 Microbial strains used

Candida albicans MRC 8908 and *C. albicans* MRC 8912 used in this study were obtained from Tygerberg Hospital, Tygerberg, South Africa. An industrial yeast strain, *Saccharomyces cerevisiae* MH1000, was obtained as a gift from the department Microbiology, Stellenbosch University, South Africa. *Saccharomyces cerevisiae* BY4742 *mnn10* and *mmn11,* both designer deletion strains deficient in N-glycosylation (Brachmann et al. 1998) were obtained from EUROSCARF (Scientific Research and Development GmbH, Bad Homburg, Germany). The cell wall mannans of these strains are compromised since the N-linked oligomannosides contain truncated poly-α1,6-mannan backbones, but the short chain O-

linked oligomannosides are unaffected (Ballou 1990). Yeast strains were stored in Yeast Malt Extract (YM) (Yarrow 1988) broth supplemented with 30% glycerol at -80°C and on YM agar slants.

Bacteroides fragilis NCTC 9343 and *Bacteroides vulgatus* ATCC 8482 were purchased from the American Type Culture Collection and stored in brain heart infusion broth (BHI; Biolab Diagnostics, Midrand, South Africa) supplemented with 30% (w/v) glycerol at -80°C, as well as on agar slants containing BHI. According to the Carbohydrate-Active Enzyme database (CAZy; http://www.cazy.org) both *Bacteroides* strains are associated with mannan degrading enzymes belonging to different glycoside hydrolases (GH) families. *Bacteroides fragilis* NCTC 9343 contains enzymes belonging to the families GH38, GH76 and GH92, all playing a role in α-mannan degradation, while *B. vulgatus* ATCC 8482 contains an α-1,2 mannosidase belonging to the family GH92.

4.2.2 Media, culture conditions and starter inoculums

For all experimentation, a quarter strength BHI broth (9.25 g/l) was used as growth medium, while the cultures were incubated at 37ºC under anaerobic conditions without shaking. In each case, these conditions were obtained with anaerobic chambers containing Anaerocult A sets (Merck, Germany) and were monitored with Anaerotest strips (Merck, Germany). Yeast inocula to be used in the experimentation were prepared in test tubes, containing 5 ml YM broth, which were each inoculated with a loop-full of growth and cultivated for 48 h under the anaerobic conditions described above. The cells were subsequently harvested via centrifugation (12 000 × *g*, 5 min) in 2 ml microcentrifuge tubes. Each resulting pellet was washed thrice with physiological saline solution (PSS; 0.89%, w/v NaCl). Yeast cells were subsequently resuspended in PSS, enumerated using a haemocytometer (Improved Neubauer, Marienfeld Superior, Germany) and then each suspension was diluted with PSS to produce the desired cell concentration. *Bacteroides* inocula were prepared in the same manner, although BHI broth was used as growth medium and the cells were enumerated using a Petroff-Hausser counting chamber (Improved Neubauer, Marienfeld Superior, Germany). Unless otherwise specified, all yeast and bacterial inocula were prepared as described above.

4.2.3 *Bacteroides* /yeast co-cultures and staining of yeasts

All five yeast strains together with the two *Bacteroides* strains were used in the experimentation. Triplicate co-culture assays were conducted in test tubes, containing a quarter strength BHI broth, each inoculated with log 6 yeast cells/ml and log 6 bacterial cells/ml. After 48 h of anaerobic incubation at 37ºC, yeast cells were enumerated using dilution plates prepared with YM agar supplemented with 200 mg/l chloramphenicol (Sigma-Aldrich, Steinheim, Germany), and incubated aerobically at 30ºC. The bacteria in each co-culture were enumerated using dilution plates prepared with BHI medium that were incubated for 48 h at 37ºC. Yeast and bacterial monocultures were included as controls in each case.

Visualization of the outer mannan layers in the yeast cell walls of selected 48 h old mono- and co-cultures was achieved using fluorescence staining. For this purpose concanavalin A, fluorescein conjugate (Life Technologies, product code C827) was used to stain *C. albicans* MRC 8908 and *S. cerevisiae* MH1000, and the cells were visualized with a Nikon Eclipse E400 epifluorescence microscope, equipped with a fluorescein isothiocyanate (FITC; excitation/barrier 465–495/515–555 nm) filter set. Images were captured with a Nikon DS-Fi2 camera and a Nikon Digital Sight DS-U3 camera controller (Nikon, Japan).

4.2.4 *Bacteroides* growth in the presence of dead yeasts

The antifungal agent, Amphotericin B (AmB) (Merck, Germany), was used to obtain intact dead cells of *C. albicans* MRC 8908 and *S. cerevisiae* MH1000. Initially, each of these strains was transferred from 48-h-old anaerobic plate cultures, prepared with YM agar, to a 2 ml microcentrifuge tube containing PSS. The resulting suspension was subsequently harvested via centrifugation (12 000 × *g*, 5 min) and hereafter the pellet (ca. 100 mg, wet weight) was suspended in 2 ml of a 6 mg/ml solution of AmB in dimethyl sulfoxide (DMSO). These suspensions contained in microcentrifuge tubes, were incubated for 4 h at 22ºC, and then again pelleted by centrifugation and treated with AmB as described above. The dead cells were washed twice by suspension in DMSO followed by centrifugation and thereafter the cells were washed with sterile distilled water (dH_2O). Finally, the dead cells were suspended in dH_2O and enumerated using a haemocytometer as described above. These cells were subsequently used as additional substrate for anaerobic monocultures of *B. fragilis* NCTC 9343 and *B. vulgatus* ATCC 8482, which were inoculated into a quarter strength BHI

broth resulting in an initial concentration of log 6 bacterial cells/ml. Each of these bacterial cultures initially either received log 3 or log 6 dead cells/ml of either dead *C. albicans* MRC 8908 or dead *S. cerevisiae* MH1000. The resulting bacterial cultures, containing the dead yeasts, were anaerobically incubated for 48 h at 37ºC, where after the bacteria were enumerated as explained above.

4.2.5 *Bacteroides* growth in the presence of dead yeasts lacking external mannan layers

Aqueous dead cell suspensions of *C. albicans* MRC 8908 and *S. cerevisiae* MH1000 were prepared as described above and the cells were pelleted using centrifugation. The mannans in the outer cell wall layers were subsequently removed by extracting twice with 6 % (w/v) NaOH for 90 min at 80ºC (Pérez and Ribas 2013). The resulting alkali-insoluble fractions, containing the dead cells with residuel cell walls consisting of chitin, β 1,3-glucan and β 1,6-glucan linked to chitin, were separated from the alkali-soluble fractions (β1,3-glucans, mannans and β1,6-glucans) via centrifugation (1 500 × *g*, 5 min). While the latter was discarded the pelleted cells were repeatedly washed with dH_2O using centrifugation, until neutral pH was reached.

The dead yeast cells, lacking mannan layers, were used as additional substrate for anaerobic monocultures of *B. fragilis* NCTC 9343 and *B. vulgatus* ATCC 8482, which were inoculated into a quarter strength BHI broth resulting in an initial concentration of log 6 bacterial cells/ml. Each of these bacterial cultures initially received log 6 dead cells/ml of either dead *C. albicans* MRC 8908 or dead *S. cerevisiae* MH1000. The resulting bacterial cultures, containing the dead yeasts, were anaerobically incubated for 48 h at 37ºC, here after the bacteria were enumerated as explained above.

4.3 Results

4.3.1 *Bacteroides* /yeast co-cultures

Bacteroides cell concentrations in the anaerobic mono- and co-cultures, after 48 h of incubation at 37ºC, are depicted in Fig. 4.2. Compared to the bacterial concentrations in the mono-cultures, *Bacteroides* numbers were significantly increased by the presence of the two *C. albicans* strains and *S. cerevisiae* MH100 in the co-cultures. Although also significant, this

increase was less pronounced in the presence of the two *S. cerevisiae mnn* deletion strains. Fluorescence staining of the outer mannan layers revealed that the presence of the bacteria resulted in no noticeable change in the cell walls of *C. albicans* MRC 8908 and *S. cerevisiae* MH100 when mono- and co-cultures were compared after 48 h (Fig. 4.3a-f). Also, no significant difference in yeast growth was observed between yeast mono- and co-cultures (Fig. 4.4).

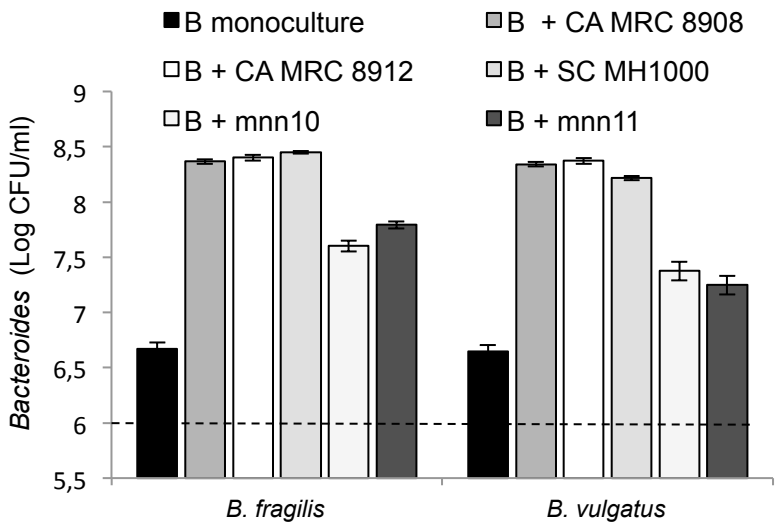

Fig. 4.2 Cell concentrations of *Bacteroides fragilis* NCTC 9343 and *Bacteroides vulgatus* ATCC 8482 when co-cultured separately with each yeast strain for 48 h at 37ºC under anaerobic conditions. B, *Bacteroides* strain; CA, *Candida albicans* strain; SC, *Saccharomyces cerevisiae* strain. *Mnn10* and *mnn11* represent the *S. cerevisiae* deletion strains. The black bars represent the controls, *Bacteroides* monocultures. Bars represent the mean of three repetitions and the whiskers indicate standard error. The dashed line represents the initial concentration of the bacteria. In all cases the co-cultured *Bacteroides* concentrations were significantly higher than that of the monocultures (Fisher LSD test, $p \leq 0.05$; Statistica Version 13, StatSoft).

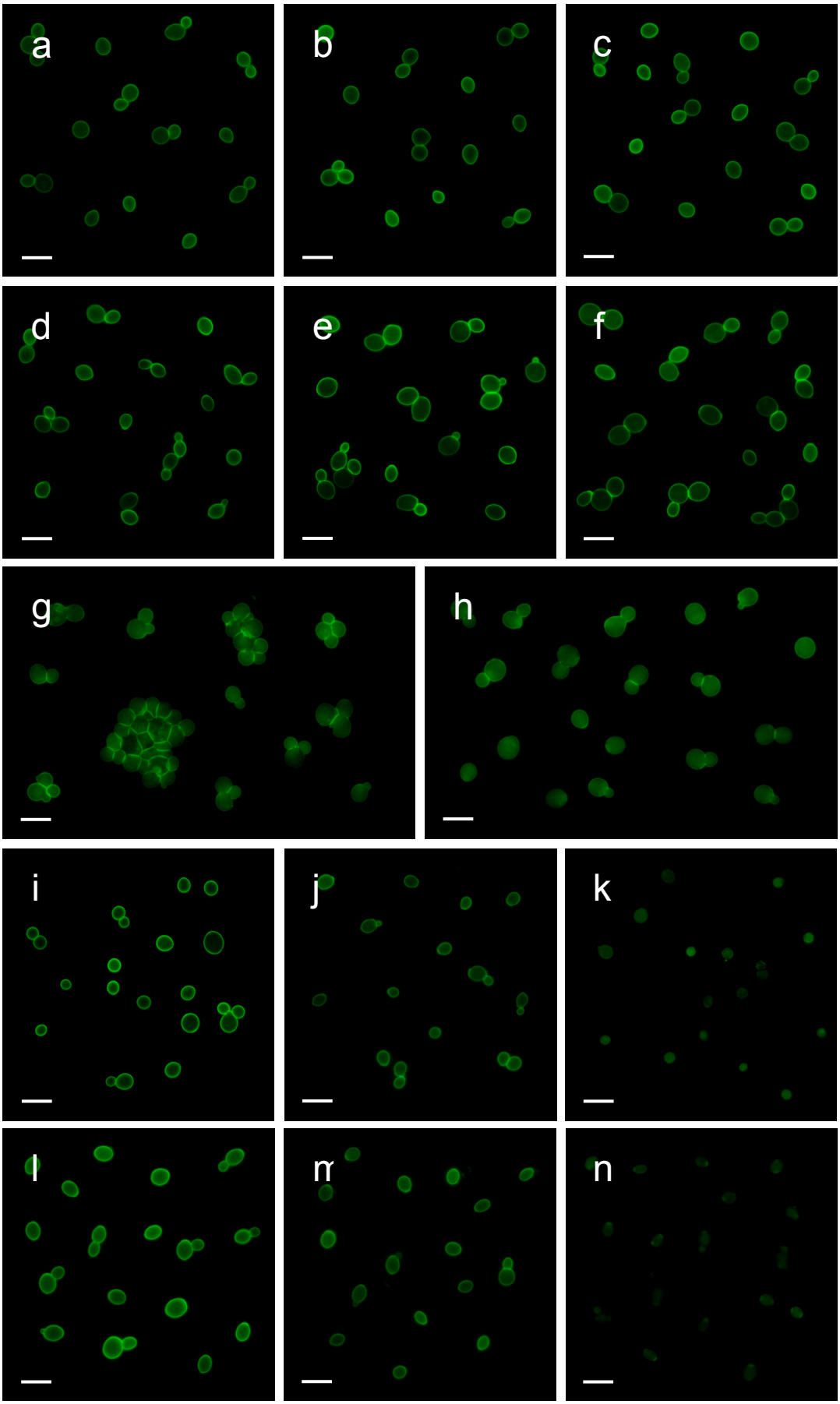

Fig. 4.3 Photomicrographs of concanavalin A, fluorescein conjugate-stained *Candida albicans* MRC 8908 (CA) and *Saccharomyces cerevisiae* MH1000 (SC) cells. **a** Live CA cells from monoculture after 48 h at 37ºC under anaerobic conditions. **b** Live CA cells when co-cultured with *Bacteroides fragilis* NCTC after 48 h at 37ºC under anaerobic conditions. No noticeable difference in the mannan layers was observed when compared to the mannan layers of yeast cells from the monocultures. **c** Live CA cells when co-cultured with *Bacteroides vulgatus* ATCC 8482 after 48 h at 37ºC under anaerobic conditions. No noticeable difference in the mannan layers was observed when compared to the mannan layers of yeast cells from the monocultures. **d** Live SC cells from monoculture after 48 h at 37ºC under anaerobic conditions. **e** Live SC cells when co-cultured with *B. fragilis* NCTC after 48 h at 37ºC under anaerobic conditions. No noticeable difference in the mannan layers was observed when compared to the mannan layers of yeast cells from the monocultures. **f** Live SC cells when co-cultured with *B. vulgatus* ATCC 8482 after 48 h at 37ºC under anaerobic conditions. No noticeable difference in the mannan layers was observed when compared to the mannan layers of yeast cells from the monocultures. **g** *Saccharomyces cerevisiae mnn10* deletion strain used as inoculum. **h** *Saccharomyces cerevisiae mnn11* deletion strain used as inoculum. **i** Live CA cells used as inoculum. **j** Dead CA cells used as inoculum. **k** Dead CA cells after treated with 6 % (w/v) NaOH for 90 min at 80ºC to remove mannan layer. No visible mannan layer can be seen. **l** Live SC cells used as inoculum. **m** Dead SC cells used as inoculum. **n** Dead SC cells after treated with 6 % (w/v) NaOH for 90 min at 80ºC to remove mannan layer. No visible mannan layer can be seen. The scale bar represents 10 μm. The low yeast cell concentrations necessitated that all images were prepared as composite images originating from a single sample.

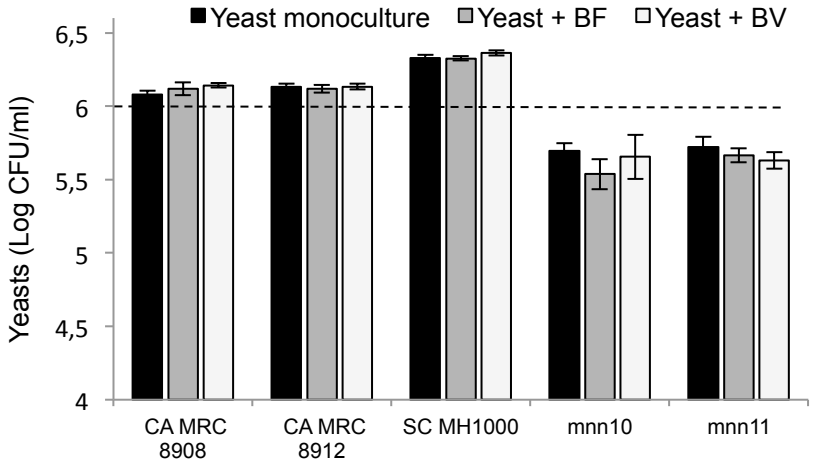

Fig. 4.4 Cell concentrations of yeast strains when co-cultured for 48 h at 37ºC under anaerobic conditions, either with *Bacteroides fragilis* NCTC 9343 (BF) or with *Bacteroides vulgatus* ATCC 8482 (BV) CA, *Candida albicans* strain; SC, *Saccharomyces cerevisiae* strain. *Mnn10* and *mnn11* represents the engineered *S. cerevisiae* strains. The black bars represent the controls, yeast monocultures. Bars represent the mean of three repetitions and the whiskers indicate the standard error. The dashed line represents the initial concentration of the bacteria. In all cases the co-cultured yeast numbers did not significantly differ from that of the monocultures (Fisher LSD test, $p > 0.05$; Statistica Version 13, StatSoft).

4.3.2 *Bacteroides* growth in the presence of dead yeasts with and without external mannan

Bacteroides growth after 48 h in the presence of dead yeasts is depicted in Fig. 4.5. It was found that dead cells of both *C. albicans* MRC 8908 and *S. cerevisiae* MH1000 exerted a similar stimulatory effect on bacterial growth. While the presence of log 3 dead yeast cells/ml resulted in a slight but significant increase in *Bacteroides* numbers, it was found that initial supplementation with log 6 dead yeast cells/ml resulted an even greater increase in *Bacteroides* numbers (Fig. 4.5). For *B. fragilis* NCTC 9343, the latter increase in bacterial numbers resulted in similar numbers than what was reached in the presence of culturable yeast cells (Fig. 4.2). In contrast, the increase in the numbers of *B. vulgatus* ATCC 8482 in the presence of log 6 dead yeast cells/ml yeasts (Fig. 4.5) was not as pronounced as was observed for this bacterial strain in the presence of culturable yeasts (Fig. 4.2). It must be

noted however, that no significant increase in culturable *Bacteroides* cell numbers was observed in bacterial cultures that were supplemented with dead yeasts of which the mannan layers were removed from the cell walls (Fig. 4.6). Removal of these mannan layers was confirmed using fluorescence staining techniques (Fig. 4.3 j,k,m,n). Photomicrographs of dead yeasts treated with heated 6 % (w/v) NaOH (Fig. 4.3 k,n), revealed the absence of mannan in the external layers of the yeasts' cell walls, when compared to intact live and dead yeast cells (Fig. 4.3 i, j, l, m).

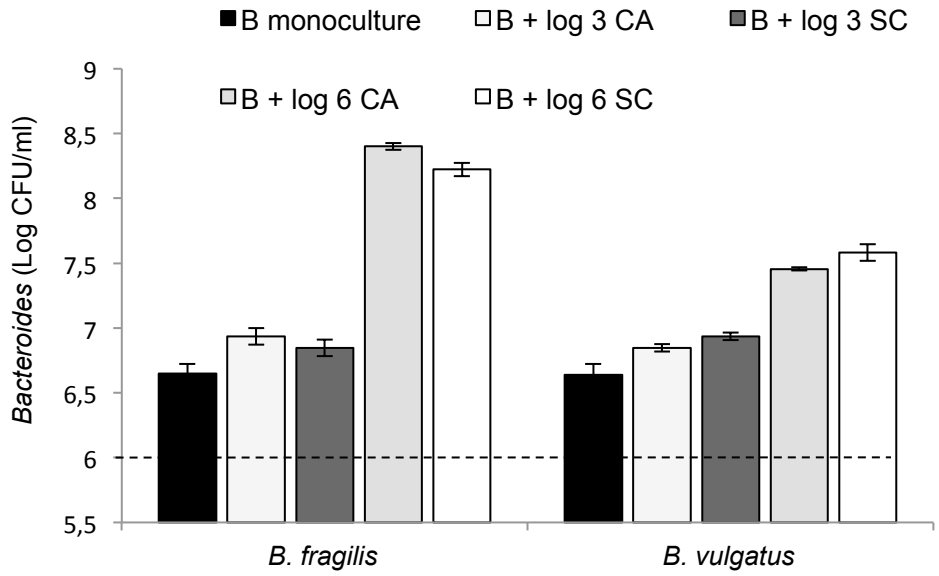

Fig. 4.5 Cell concentrations of *Bacteroides fragilis* NCTC 9343 and *Bacteroides vulgatus* ATCC 8482 when cultured separately with either log 3 or 6 dead yeast cells/ml of *Candida albicans* MRC 8908 (CA) or *Saccharomyces cerevisiae* MH1000 (SC) for 48 h at 37ºC under anaerobic conditions. B, *Bacteroides*. The black bars represent the controls, *Bacteroides* monocultures. Bars represent the mean of three repetitions and the whiskers indicate the standard error. The dashed line represents the initial concentration of the bacteria. The presence of log 3 dead yeast cells/ml resulted in a slight increase in *Bacteroides* concentrations, nevertheless *Bacteroides* concentrations in presence of both log 3 and log 6 dead yeast cells/ml were significantly higher than that of the controls (Fisher LSD test, $p \leq 0.05$; Statistica Version 13, StatSoft).

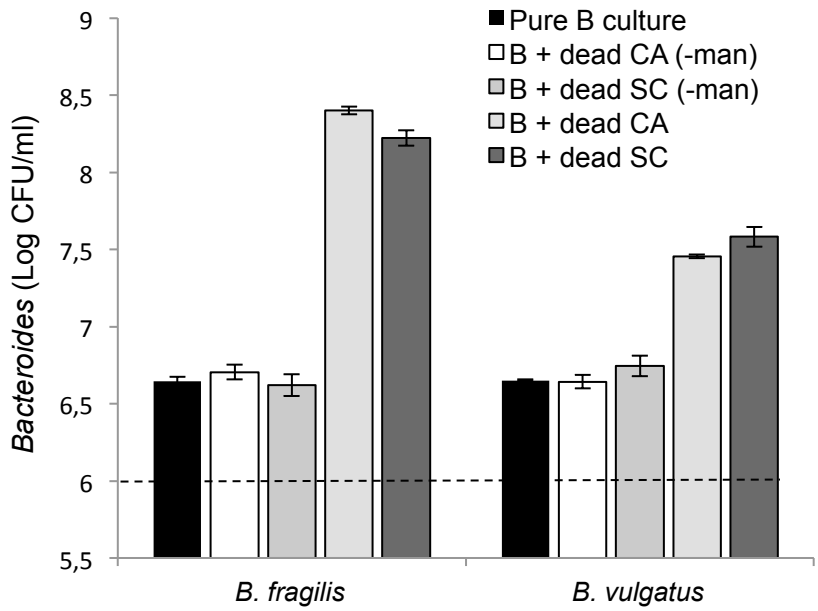

Fig. 4.6 Cell concentrations of *Bacteroides fragilis* NCTC 9343 and *Bacteroides vulgatus* ATCC 8482 when cultured separately with log 6 dead yeast cells/ml of *Candida albicans* MRC 8908 (CA) or *Saccharomyces cerevisiae* MH1000 (SC) and with log 6 dead yeast cells/ml after the mannan layer of both *C. albicans* (-man) and *S. cerevisiae* (-man) was removed by alkali extraction. B, *Bacteroides*. The black bars represent the controls, *Bacteroides* monocultures. Bars represent the means of three repetitions and the whiskers indicate the standard error. The dashed line represents the initial concentration of the bacteria. In all cases the *Bacteroides* cultured with dead yeast cells without a mannan layer did not significantly differ from that of the control (Fisher LSD test, $p > 0.05$; Statistica Version 13, StatSoft). However, *Bacteroides* cultured with the intact dead yeast cells with mannan layer still present, showed significantly more growth after 48 h compared to the control monocultures (Fisher LSD test, $p \leq 0.05$; Statistica Version 13, StatSoft).

4.4 Discussion

Representatives of *Bacteroides* (*B. fragilis* NCTC 9343 and *B. vulgatus* ATCC 8482) a genus commonly associated with the human GI tract (Kraal et al. 2014), showed significantly more growth in anaerobic co-cultures with *C. albicans* MRC 8908, *C. albicans* MRC 8912 and *S. cerevisiae* MH1000 than in their corresponding monocultures (Fig. 4.2). These wild type ascomycetous yeasts, which also represented species that may occur in the human GI tract

(Pecquet et al. 1991; Moran et al. 2012), stimulated *Bacteroides* growth significantly more than the two engineered yeast strains (*S. cerevisiae mnn10* and *S. cerevisiae mnn11*; Fig. 4.2) containing reduced levels of cell wall mannan (Fig. 4.3g, h).

Since *B. fragilis* NCTC 9343 and *B. vulgatus* ATCC 8482 are known to possess mannan degrading enzymes, i.e. α-mannosidase and α-mannanase (El Kaoutari et al. 2013) (CAZy; http://www.cazy.org) and it was previously demonstrated that *Bacteroides* species are able to utilize yeast mannan (Cuskin et al. 2015), the above-mentioned results indicated that growth of *Bacteroides* within the *Bacteroides*/yeast co-cultures was enhanced by mannan in the yeast cell walls. Further evidence for the positive effect of yeast outer mannan layers on *Bacteroides* growth was obtained when the two bacterial strains were cultured in the presence of dead cells of both *C. albicans* and *S. cerevisiae*. It was found that although intact dead yeast cells stimulated *Bacteroides* growth (Fig. 4.5), this enhanced bacterial growth observed in the presence of dead yeasts did not occur when the *Bacteroides* strains were cultured in the presence of dead yeasts without outer mannan layers (Fig. 4.6). Interestingly, after 48 h of incubation in the presence of log 6 intact dead yeasts/ml *B. fragilis* NCTC 9343 displayed significantly more growth than *B. vulgatus* ATCC 8482. It is tempting to speculate whether this may be the result of *B. vulgatus* ATCC 8482 displaying only α-1,2 mannosidase activity (CAZy; http://www.cazy.org), while *B. fragilis* NCTC 9343 have both α-1,2 mannosidase activity, as well as α-1,6 mannanase activity (CAZy; http://www.cazy.org) enabling it to also act on the α-1,6 mannan backbone (Fig. 4.1) and utilize more of the yeast mannans.

In contrast to the bacterial response to the presence of yeast cells, none of the yeast strains, including the engineered strains with compromised mannan layers, seemed to have been significantly affected by the presence of the bacteria in the anaerobic *Bacteroides*/yeast co-cultures (Fig. 4.4). Similar results were previously obtained for anaerobic bacteria/yeast co-cultures of *C. albicans* and a number of bacteria representing Firmicutes and Proteobacteria (Benadé et al. 2015). It was found that under anaerobic conditions candidial growth persisted even in the presence of bacterial extracellular mannan degrading enzymes. This phenonmenon and our finding that yeast cell wall mannan layers were unaffected by the presence of mannan utilizing *Bacteroides* strains (Cuskin et al. 2015; El Kaoutari et al. 2013) in anaerobic co-cultures (Fig. 4.3a-f) highlighted the robustness of mannan layers in the cell wall of these ascomycetous yeasts. It would therefore appear that the mannans of *C. albicans*

and *S. cerevisiae* were being replenished as the *Bacteroides* strains were "exfoliating" the yeast cells under these anaerobic conditions at 37°C.

While it has been shown that *S. cerevisiae* may occur in the human GI tract (Pecquet et al. 1991; Scevola et al. 2013), it is well-known that *C. albicans* is a commensal, forming part of the human GI tract microbiome of almost all healthy individuals (Moran et al. 2012). It is generally thought that this pleomorphic yeast occurs in its yeast form within the human GI tract (White et al. 2007), containing notably more cell wall mannan compared to its hyphal form (Machová et al. 2015; Staniszewska et al. 2013).

Even though it was previously suggested that *Bacteroides* species, occurring in the human GI tract, have adapted to ulitize yeast mannans originating from ascomycetous yeasts, this is the first report demonstrating enhanced growth of *Bacteroides* species in the presence of cultureable ascomyceous yeasts. Our findings thus suggest that under these anaerobic conditions *C. albicans*, and even *S. cerevisiae,* may potentially form symbioses with mannan utilizing commensalists of the genus *Bacteroides.* The potential implications of this symbiosis on human health are unclear and should therefore be studied in more detail.

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Chapter 5

General Conclusions and Future Research

General Conclusions and Future Research

5.1 General conclusions

The ascomycetous yeast *Candida albicans* (Robin) Berkhout (1923) is a pleomorphic fungus (Chester and Cooper 2011; Moran et al. 2012) and is the most common human fungal pathogen (Beck-Sagué and Jarvis 1993; Chester and Cooper 2011). It can live as a harmless commensal in many anatomical sites within a mammalian host. The potential colonisable sites include; the gastrointestinal (GI) and genitourinary tracts, oral cavities and skin of healthy individuals, without causing significant infections (Chester and Cooper 2011). However, endogenous *C. albicans* infections are triggered by a compromised immune system, or when the competing microbiota are eliminated for example after antibiotic administration (Wilson et al. 2012). *Candida albicans* is one of the few microbial pathogens having the potential to cause infection at such an extensive range of diverse niches within its host (Calderone and Fonzi, 2001).

This pathogen is able to withstand significant changes in oxygen and carbon dioxide levels, pH, osmolarity, temperature, and availability of nutrients (Kumamoto 2008). These characteristics could therefore enable the yeast to thrive in external environments. However, the probability that *C. albicans* may exist in environmental niches, away from its mammalian host, is not generally considered during studies of the natural history of this yeast. Hube (2004) stated that it is surprising that *C. albicans* is exclusively associated with animals or humans and hardly found in environmental niches such as soil, since it has no specific nutrient requirements that would prevent it from surviving in the outside environment. Yet, *C. albicans* strains are occasionally isolated from sewage sludge and waste waters contaminated with fecal material (Buck and Bubucis 1978; Cook and Schlitzer 1981; Cooke et al. 1960). A study done by Stone et al. (2012) demonstrated that *C. albicans* is capable of growth along the banks of a polluted river. Using quantitative real-time PCR they found *C. albicans* to be capable of sustainable growth in oxygen limited zones, while no evidence of such growth for this yeast was obtained in the more aerobic zones of the river. The reason and mechanisms allowing *C. albicans* to survive in the oxygen limited zones and not in the free flowing aerobic oxidizing zones, was still unclear.

During this study, co-culture experimentation at 26ºC between several phylogenetic unrelated bacterial species and *C. albicans,* revealed that the bacteria had a negative effect on *C. albicans* growth under aerobic conditions, while this effect was ameliorated under anaerobic conditions (Chapter 2). The bacteria used in the experimentation represented *Aeromonas hydrophila*, *Bacillus cereus*, *Bacillus subtilis*, *Clostridium, Enterobacter*, *Klebsiella pneumonia, Kluyvera ascorbata* and *Serratia marcescens*. Subsequent, enzyme assays revealed that the majority of the bacterial strains used in the experimentation aerobically produced extracellular hydrolytic enzymes capable of yeast cell wall hydrolysis, including chitinases and mannan degrading enzymes. These findings supported the results of others who demonstrated that a wide variety of bacteria are mycolytic and produce hydrolytic enzymes capable of degrading the different components of the fungal cell wall (Azam and Malfatti 2007; De Boer et al. 2005), which is known to be an important protective structure of microorganisms including yeasts (Bowman and Free 2006). It must be noted however, that except for one bacterial strain representing *A. hydrophila,* the above-mentioned cell wall degrading enzymes were not detected in the anaerobic bacterial cultures that were analysed during this study (Chapter 2). In addition, the antimicrobial compound prodigiosin, produced by *S. marcescens* was detected under aerobic conditions only. Previous research indicated that prodigiosin and chitinase exert a synergistic antifungal effect on *Botrytis cinerea* by inhibiting spore germination (Someya et al. 2001). Our results indicated that bacterial mannan degrading enzymes have a greater synergistic effect in combination with the antimicrobial compound prodigiosin, than chitinases, in controlling *C. albicans* growth. Also this enhanced synergistic effect was only observed under aerobic conditions (Chapter 2). These results may provide an indication why *C. albicans* would be able to survive in oxygen limited aquatic zones (Stone et al. 2012), but not in aerobic zones found in the external environment away from an animal host.

In the GI tract of humans, *C. albicans* population size is thought to be regulated by commensal bacteria (Kennedy and Volz 1985). Hence, our results obtained with anaerobic bacteria/yeast co-cultures at 26ºC may contribute to knowledge of how this yeast survives in the presence of bacteria within its human host (Chapter 2). These findings were supported by the results obtained with anaerobic bacteria/yeast co-cultures at 37ºC (Chapter 3). Under the latter conditions, it was found that growth of *C. albicans* was unaffected by the presence of the bacterial strains used in this experimentation. The latter included anaerobic and facultative anaerobic bacteria that may occur in the human GI tract and represented the

species *Bacteroides fragilis, Bacteroides vulgatus, Clostridium perfringens, Escherichia coli*, *K. pneumoniae, Lactobacillus acidophilus* and *Lactobacillus plantarum*, (Eggerth and Gagnon 1933; Finegold et al. 1974; Moore and Holdeman 1974; Rajilić-Stojanović et al. 2007; Rajilić-Stojanović 2007).

Since *C. albicans* remains in the yeast phase while being cultivated under anaerobic conditions at 37ºC, plate counts could be used to determine and compare yeast growth (Chapter 3). Under aerobic conditions at 37ºC however, *C. albicans* mostly grows in its hyphal phase (Sudbery 2011; Chapter 3) rendering it difficult to directly compare anaerobic and aerobic growth of *C. albicans* under these conditions. Nevertheless, during this study (Chapter 3) fluorescence microscopy was employed to estimate yeast viability in the aerobic bacteria/yeast co-cultures. Photomicrographs of yeasts after being co-cultured with the facultative anaerobic bacteria showed some dead cells and indicated that *C. albicans* may be less resistant to these bacteria under aerobic conditions than under anaerobic conditions. These results supported previous findings (Chapter 2), which revealed that the representative of *K. pneumoniae* is able to kill yeast cells of *C. albicans* under aerobic conditions at 26ºC. In addition, similar to previous findings on the ability of *K. pneumoniae* to produce cell wall degrading enzymes at 26ºC (Chapter 2), this bacterium was also found to produce mannanases at 37ºC (Chapter 3). However under anaerobic conditions at 37ºC, and in contrast to observations previously conducted under aerobic conditions at 26ºC (Chapter 2), the presence of bacterial cell wall degrading mannanases did not seem to enhance the antagonistic effect of antimicrobials such as Amphotericin B and prodigiosin.

In general, the bacteria used in this study were not affected by the presence of the ascomycetous yeast *C. albicans,* neither at 26 or 37ºC, nor under aerobic and anaerobic conditions (Chapter 2 and 3). However, under anaerobic conditions at 37ºC the numbers of the two *Bacteroides* strains, representing *B. fragilis* and *B. vulgatus,* were significantly increased by the presence of *C. albicans* (Chapter 3 and 4). When these bacteria, known to utilize mannan present in the ascomycetous yeast cell wall (Cuskin et al. 2015), were co-cultured with yeast strains representing another ascomycetous yeast, *Saccharomyces cerevisiae*, a similar increase in bacterial numbers was observed (Chapter 4). Co-culturing with engineered *S. cerevisiae* strains with compromised cell wall mannan layers however, resulted that the increase in bacterial growth was not as pronounced as when bacteria were co-cultured with a wild type *S. cerevisiae* strain. Treating monocultures of the two

Bacteroides species with intact dead cells belonging to either *C. albicans* or *S. cerevisiae* also resulted in significant increases in bacterial numbers. However, treatment with dead yeast cells of which the cell wall mannan layers were removed with alkali extraction, showed no increase in *Bacteroides* numbers. Since the culturable yeast numbers were unaffected by the presence of the *Bacteroides* strains, it can be concluded that under anaerobic conditions mannan utilizing representatives of the genus *Bacteroides* may form commensalistic interactions with ascomycetous yeasts such as *C. albicans* and *S. cerevisiae.*

To conclude, by studying binary interactions between bacteria and *C. albicans* it was found that this yeast survived better under anaerobic conditions in the presence of bacteria compared to under aerobic conditions. The phenomenon was ascribed to reduced production of bacterial antimicrobial compounds such as prodigiosin, as well as bacterial cell wall degrading enzymes such as chitinases and mannannases. The cell wall of anaerobically grown *C. albicans* also seemed to be more resistant to bacterial mannannases, since in contrast to the findings obtained under aerobic conditions; bacterial cell wall degrading mannanases did not seem to enhance the antagonistic effect of antimicrobials, such as Amphotericin B and prodigiosin, on *C. albicans* that was cultured under anaerobic conditions. Likewise, the cell wall mannan layers of anaerobically cultivated ascomycetous yeasts such as *C. albicans* and *S. cerevisiae* were found to be unaffected by the presence of mannan utilizing *Bacteroides* strains, thus highlighted the robustness of these mannan layers in the yeast cell wall under these conditions. The findings suggested that under the anaerobic conditions occurring in the human GI tract these ascomycetous yeasts may potentially form symbioses with mannan utilizing commensalists of the genus *Bacteroides.*

5.1 Future research

Although evidence for mannan dependent symbioses between selected bacteria and *C. albicans* was obtained by analysing *in vitro* binary interactions during this study, it is envisaged that in future these interactions will be studied in more complex ecosystems. Within the external natural environment, as well as in the human GI tract, *C. albicans* may simultaneously interact with a number of bacteria. Thus, to better understand complex bacteria/yeast interactions in such environments temporal response dynamics of microbial communities within these environments should be studied using metagenome high throughput sequencing, as well as proteonomics. The results may provide a more detailed

view of the potential interactions between *C. albicans* and *Bacteroides* species, by revealing changes in the relative abundance of these taxa, as well as the proteins involved in their interactions within the human GI tract. Knowledge of the complex interactions between *C. albicans* and the bacteria in the human GI tract may provide a better understanding of the role of bacteria in the pathogenicity of this yeast.

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Appendix:

Supplementary material

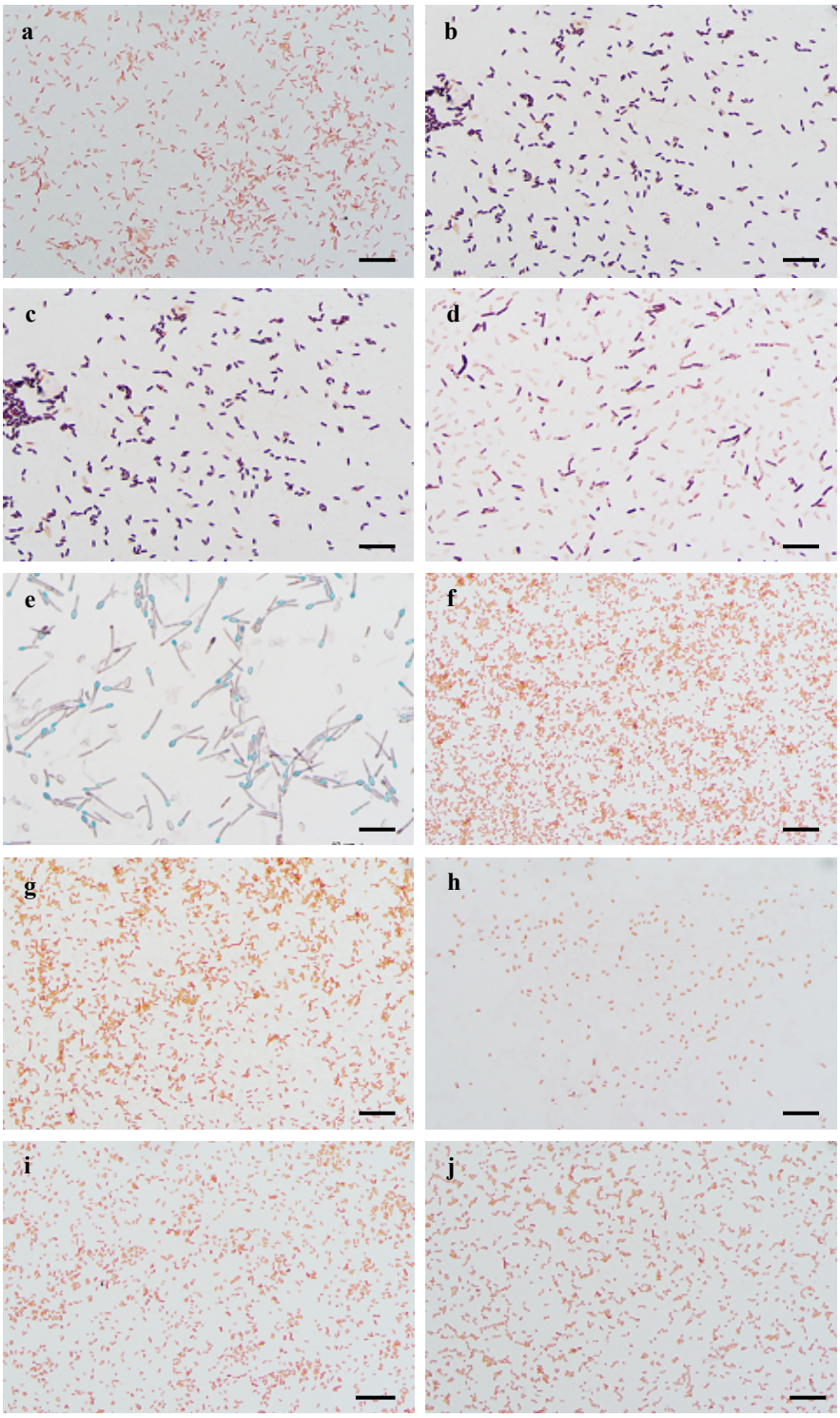

Fig. S1 Light microscopy images of Gram stained bacterial isolates. **a** *Aeromonas hydrophila*; **b** *Bacillus cereus*; **c** *Bacillus subtilis*; **d** *Clostridium* sp 1115; **e** *Clostridium sp* 1116; **f** *Enterobacter ludwigii*; **g** *Enterobacter* sp 1098; **h** *Klebsiella pneumonia*; **i** *Kluyvera ascorbata*; **j** *Serratia marcescens*. Light microscopy images were conducted using a Nikon eclipse E400 microscope equipped with a Nikon DS-Fi2 camera and a Nikon Digital Sight DS-U3 camera controller (Nikon, Japan). The scale bar represents 10 µm.

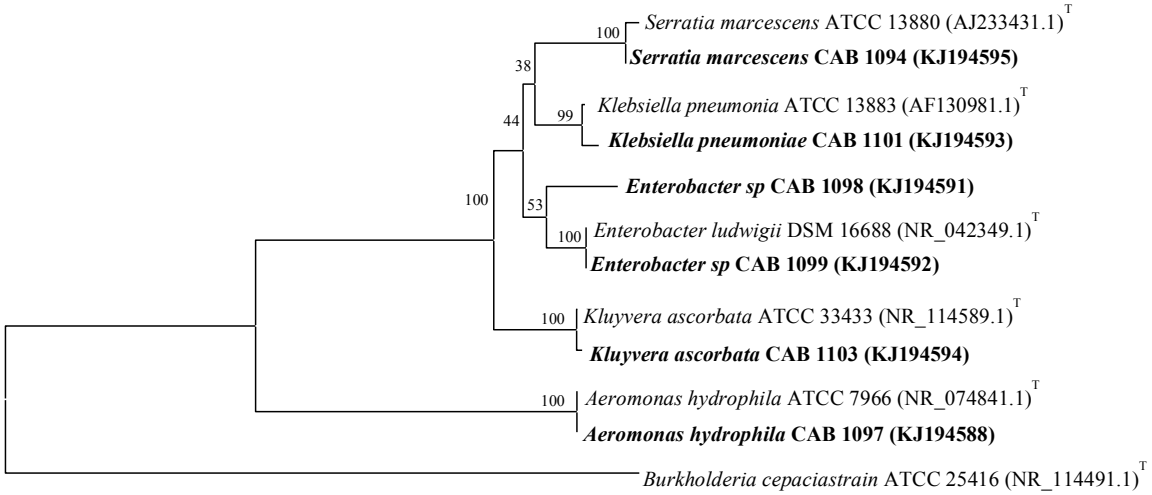

Fig. S2 Phylogenetic tree of 16s rRNA gene sequences of Proteobacteria. T Type strains; Boot strap 1000

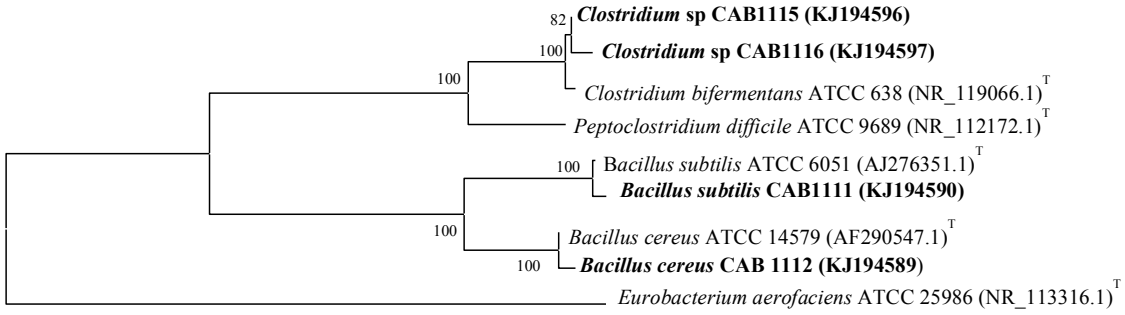

Fig. S3 Phylogenetic tree of 16s rRNA gene sequences of Firmicutes .T Type strains; Boot strap 1000

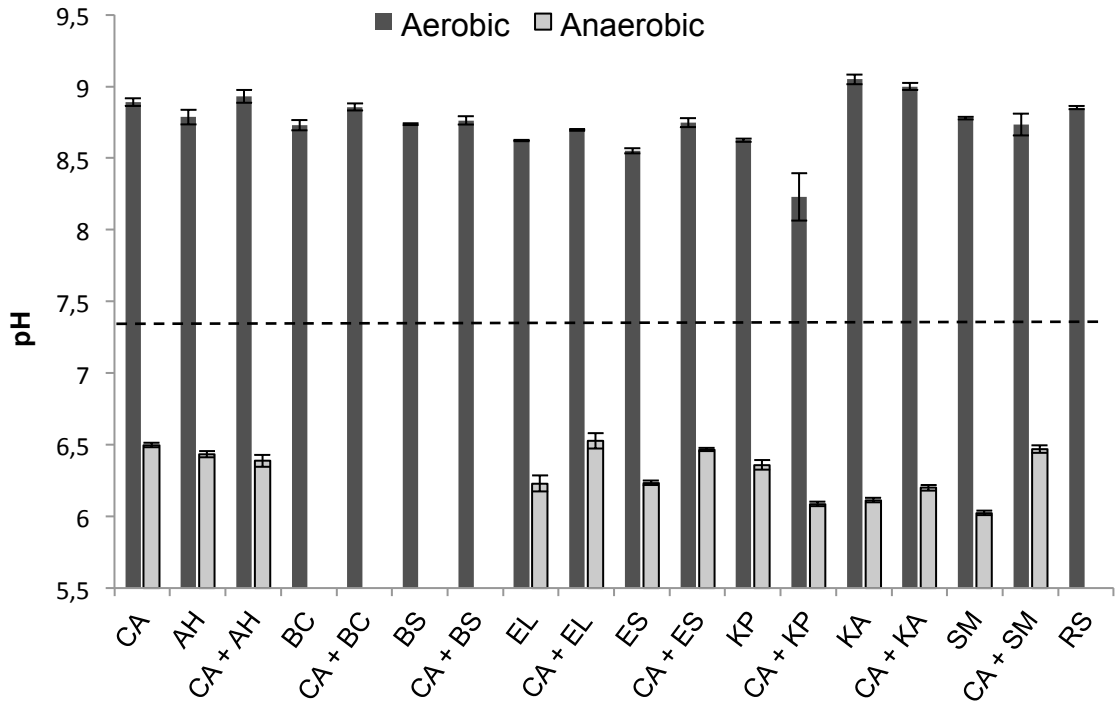

Fig. S4 pH of mono- and co-cultures after incubation at 26ºC for 72 h. The pH of all aerobic cultures (co-cultures and monocultures) increased from 7.4 to ca. 8.5 after 72 h of incubation. In contrast, the pH of all the anaerobic cultures decreased to ca. pH 6.5. CA, *Candida albicans* CAB 1084; AH, *Aeromonas hydrophila* CAB 1097; BC and BS, *Bacillus* isolates (CAB 1112 and CAB 1111); EL and ES, *Enterobacter* isolates (CAB 1099 and CAB 1098); KP, *Klebsiella pneumoniae* CAB 1101; KA, *Kluyvera ascorbata* CAB1103; SM, *Serratia marcescens* CAB1094. The dash line represents the initial pH of the media.

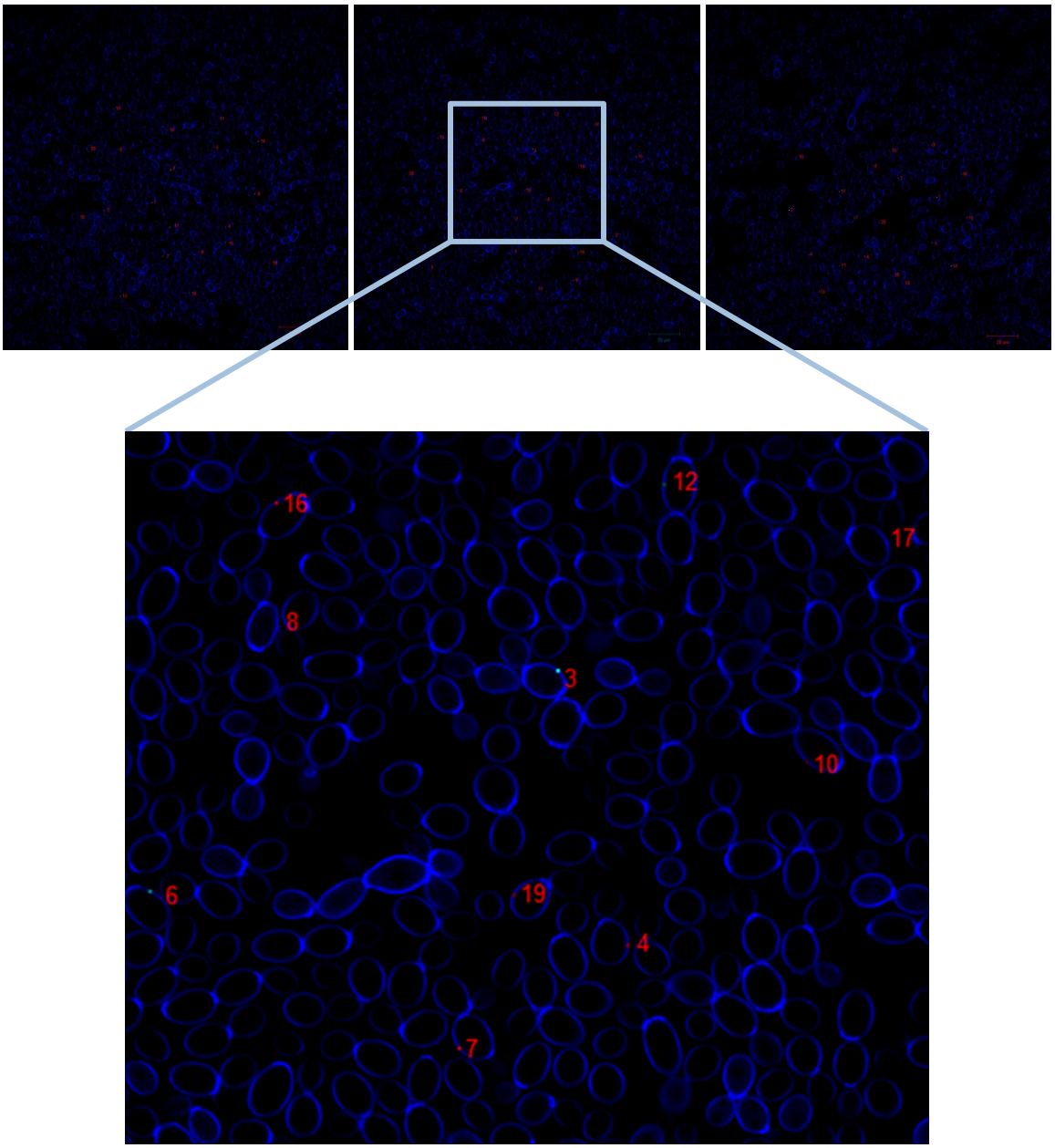

Fig. S5 The 60 randomly selected aerobic *Candida albicans* cells picked to determine relative fluorescent intensity after staining cells with calcofluor white. Spots were never selected near yeast bud scars due to the higher chitin content in bud scars (Cabib and Bowers, 1971). Fluorescence microscopy conducted with a Carl Zeiss Confocal LSM 780 Elyra S1 and SR-SIM superresolution platform was used to capture cellular images. Relative fluorescence intensity of the yeast cell walls were determined using ZEN microscope software (version 2011) (Germany).

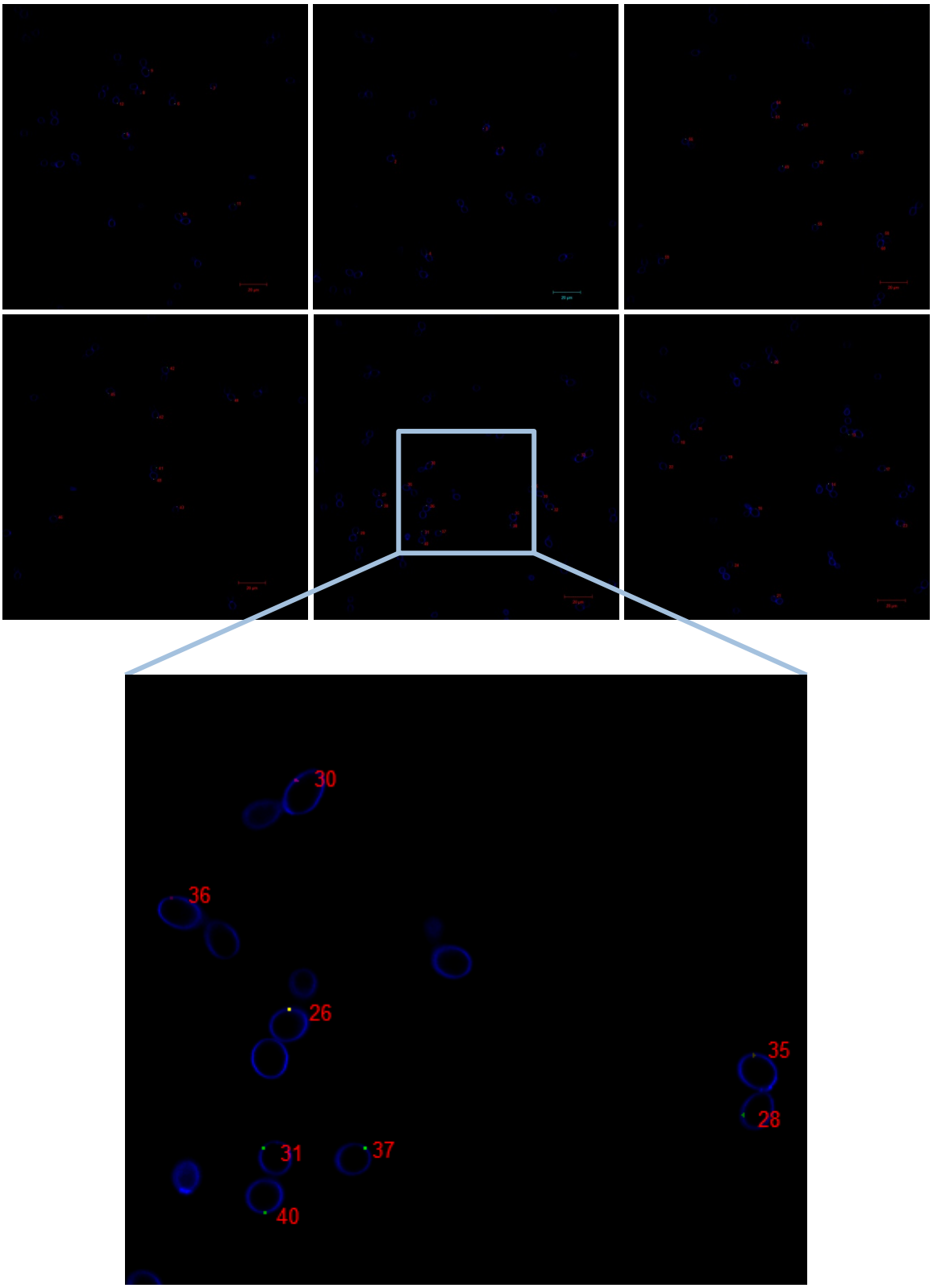

Fig. S6 The 60 randomly selected anaerobic *Candida albicans* cells picked to determine relative fluorescent intensity after staining cells with calcofluor white. Spots were never selected near yeast bud scars due to the higher chitin content in bud scars (Cabib and Bowers,

1971). Fluorescence microscopy conducted with a Carl Zeiss Confocal LSM 780 Elyra S1 and SR-SIM superresolution platform was used to capture cellular images. Relative fluorescence intensity of the yeast cell walls were determined using ZEN microscope software (version 2011) (Germany).

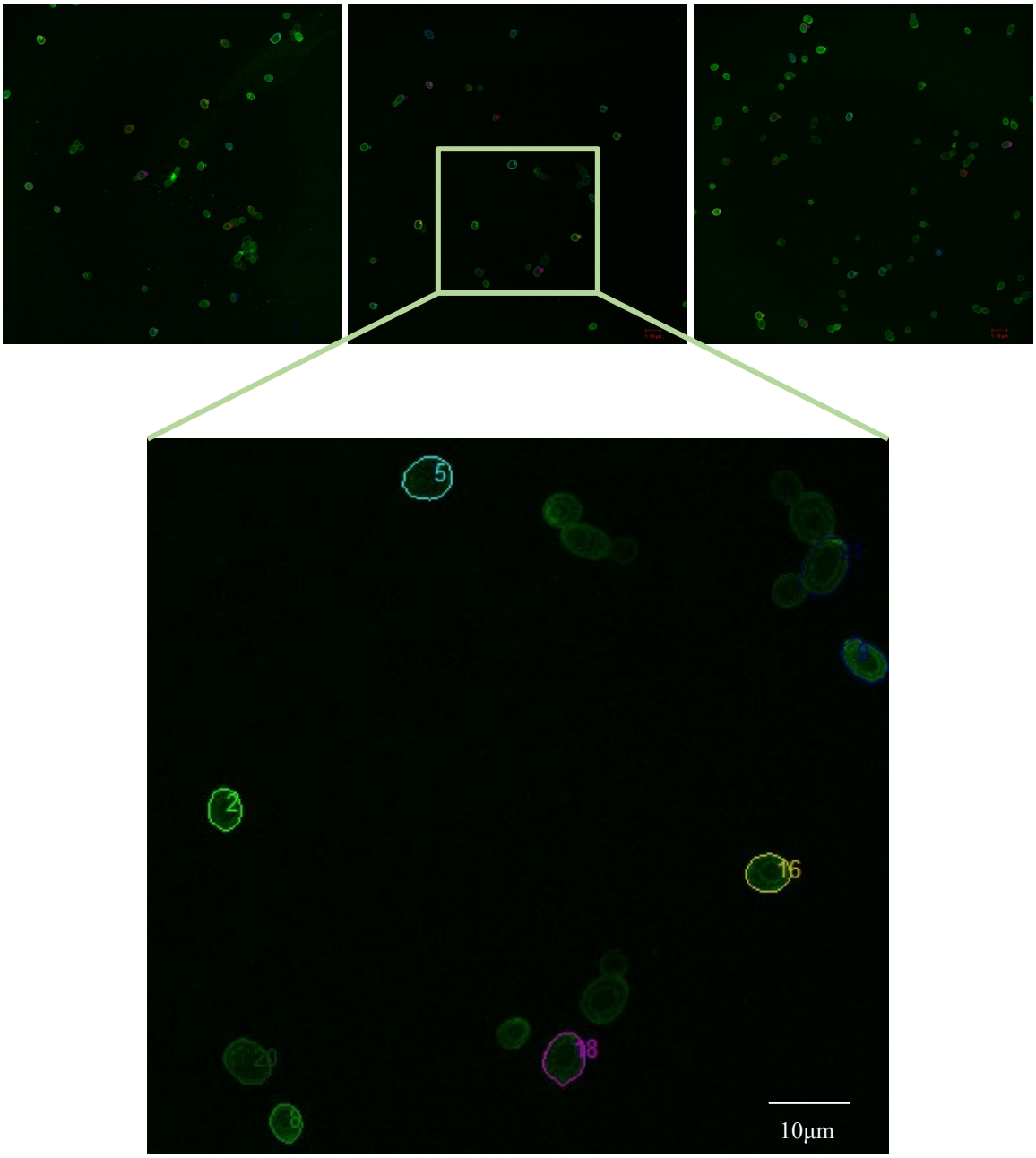

Fig. S7 The 60 randomly selected aerobic *Candida albicans* cells picked to determine the relative fluorescence intensity of the entire mannan layer after staining with concanavalin A, fluorescein conjugate. Fluorescence microscopy conducted with a Carl Zeiss Confocal LSM 780 Elyra S1 and SR-SIM superresolution platform was used to capture cellular images.

Relative fluorescence intensity of the yeast cell walls were determined using ZEN microscope software (version 2011) (Germany).

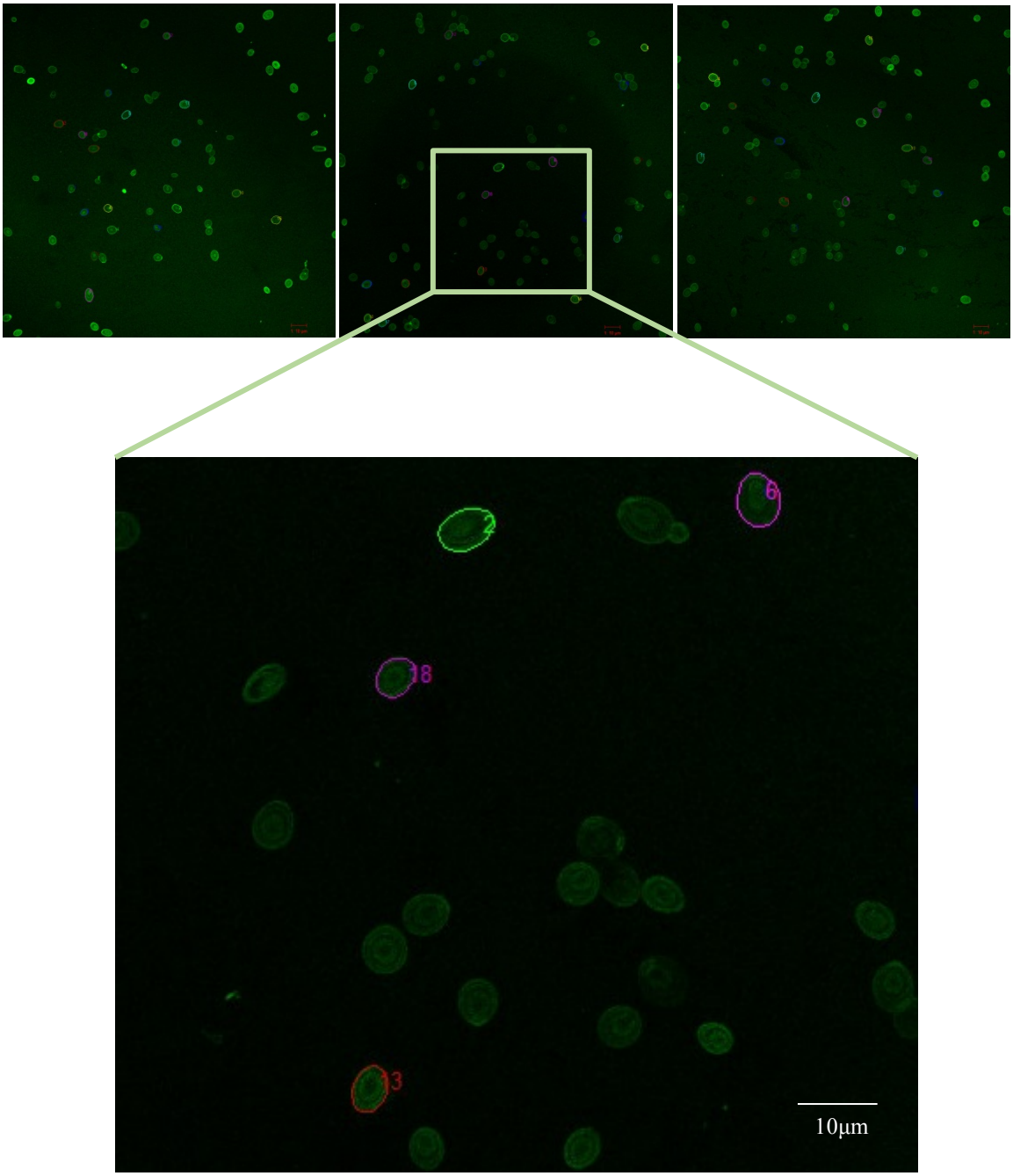

Fig. S8 The 60 randomly selected anaerobic *Candida albicans* cells picked to determine the relative fluorescence intensity of the entire mannan layer after staining with concanavalin A, fluorescein conjugate. Fluorescence microscopy conducted with a Carl Zeiss Confocal LSM

780 Elyra S1 and SR-SIM superresolution platform was used to capture cellular images. Relative fluorescence intensity of the yeast cell walls were determined using ZEN microscope software (version 2011) (Germany).